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High pressure liquid chromatography ion exchange studies on bile relating to the postmortem interval : a thesis ...

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HIGH PRESSURE LIQUID CHROMATOGRAPHY ION EXCHANGE STUDIES ON BILE RELATING TO THE POSTMORTEM INTERVAL

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by

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CHAPTER I

INTRODUCTION

Determination of the postmortem interval is often a necessary activity in our complex society. Examples of situations in which legal officials would be particularly concerned with this information are: fatal traffic accidents with a body recovered from the road or railroad track, suspected homicide...especially when suicide or death by natural causes is feigned or the account given by the accused does not fit the general picture, and suicide disguised as natural death. Even apart from the realm of forensics, absolute importance is placed upon time of death for the assignment and distribution of estate. The need for this information is evident by the fact that all standard forms of death certificate require a statement as to time of death.

In light of the importance of this determination, specialists in medico-legal practice should have at their disposal a set of tests by which they could estimate time of death as exactly as possible. There is, in fact, a whole range of tests published or conventionally being used, relating physiological or biochemical changes to time of death in the early postmortem interval when the body as a

whole is still more or less unimpaired by decomposition (about one week under usual circumstances). The majority of cases by far fall within this time frame. However, even the most promising of tests show insufficient accuracy to be used alone, and examiners usually rely on a battery of tests to increase the certainty of their estimations. Beyond one and a half to two days postmortem the number of applicable tests falls off drastically, leaving the remaining days of the early postmortem period beset with the greatest difficulties in assesing time of death. Clearly a need remains for another assay or test useful in the estimation of time of death after day two of this time period.

Selected characteristics of an assay or test will determine its usefulness in a medico-legal laboratory. Considerations for a suitable test to estimate time of death include selection of the particular change to be observed, choice of sample, and the establishment of a workable means of measuring the change. Biochemical changes usually quantify well. By the second day and with successive days after death one would expect to find measurable increases of decomposition products. In the putrefaction of protein, concentrations of ammonia and certain small carboxylic acids should increase. A fluid rather than solid tissue would facilitate assay; this fluid should be fairly easy to extract without fear of contamination and come preferably from an isolated system whose barriers remain quite intact

during the early postmortem interval. Finally, one must find a means of measurement that is sufficiently quantitative and reproducible. Ideally either the equipment for this measurement should be universally available or the sample should be sufficiently stable for transport to a properly equipped facility. All these considerations were kept in mind for the selection of a proposed new test.

It is the purpose of this study, then, to investigate the feasibility of using the changes of concentrations of small ionic decomposition products in bile as indicators of time since death. High pressure liquid chromatography was selected for measurement of these changes.

CHAPTER II

A REVIEW OF THE LITERATURE AND PROCEDURES

Established tests applicable to determination of the early post mortem interval could be grouped into three major categories: classic methods of inspection, supravital reactions, and biochemical changes.

Among the classic observations, body temperature and the presence or absence of rigor mortis are the most well known.

Dieckmann (1961) describes the common method of evaluating the postmortem interval by body temperature. An investigator at the scene of the discovery of a body will immediately make a preliminary, however coarse, evaluation of body temperature, placing a hand to a protected area, usually an arm pit. If the body is warm to touch, he would assume that death occurred within just a few hours; if cold and clammy, within eighteen to twenty-four hours or more. Researchers have attempted to better quantify this observation, realizing that the cooling rate greatly and decisively depends upon the whole of environmental conditions as well as the body size and site of temperature measurement. In a review

of measurement of body temperature for the estimation of postmortem interval Schleyer (1963) determined that the best correlation between temperature and time of death was achieved by those investigators who had constructed a data curve from numerous known cases, maintaining a consistent site for measurement. Repeated temperature readings improved correlation. Best results were achieved by maintaining the body under strictly constant environmental conditions during the entire time of observation and then subjecting the values obtained to mathematical treatment. While the mathematic formulae used by different investigators varied some, those yielding the most consistent results according to Schleyer's observations (1963) demonstrated a cooling curve of a "logarithmic type". These more careful data may be reflecting the fact that the rate of change of body temperature is also changing with time as the body temperature approaches the temperature of the environment. Useful correlation of this measurement with the time since death is obtained only within a rather narrow environmental temperature range, usually plus or minus three centigrade degrees. Expected error under even the best of conditions is plus or minus one hour, and the outside limit of applicability is thirty-six hours postmortem.

Schleyer (1963) notes that until the middle of this

century the order and speed of the spread of rigor mortis and its disappearance was regarded as dogma. Investigators now realize that the pattern is by no means as regular as previously expected; in fact, both exterior and interior factors can cause considerable variation in the time schedule. Schleyer (1963) describes the establishment of rigor mortis as a simultaneous event, that is to say, all muscle tissue begins to transform at the same time, with complete involvement of small muscles being evident sooner than that of the large, giving an apparent "top to bottom" progression throughout the body. Dieckmann (1961) gives a typical time pattern showing involvement of the small muscles in one to two hours, and face and jaw muscle in three to five hours. In large muscle, rigor is complete within six to eighteen hours. After another three to four hours, rigor begins to disappear from the upper half of the body. The disappearance follows pretty much a reverse of the onset, with complete loss of rigor by twenty-nine to thirty-four hours postmortem. According to Dieckmann (1961), even this relatively short span of applicability loses usefulness when the establishment of rigor is beset with complicating influences. Onset will take place more rapidly when the deceased has been involved in intense emotional or physical activity immediately prior to death. On the other hand, rigor

mortis will proceed more slowly in the weak, feeble, or exhausted, or in those persons showing unusually heavy musculature.

A number of other observations are available to the professional, giving additional clues as to the time of death. Schleyer (1963) claims that livor mortis, purplish discoloration of the lower parts of the body resulting from gravitational settling of blood, becomes evident by about eight hours after death and is impossible to remove with moderate finger pressure by ten to twelve (some say as much as twenty) hours postmortem. Examination of the contents of stomach and small intestine can be helpful if the habits of the deceased or time and content of the last meal are known. Normally the stomach will have more or less emptied within two to eight hours after ingestion. Night deaths with full urinary bladder indicate death probably took place in the second half of the night; an empty bladder is a useful clue only if postmortem effusion can be ruled out. All these observations are useful only within the time frame of one day.

Schleyer (1963) also reviews the postmortem interval predictive value of certain supravital reactions, those reactions showing the sensitivity of human tissues to various stimuli for a brief time after death. Reactions of muscle tissue are most often

observed, and stimuli may be mechanical, electrical or pharmacological. Regular contractions of hand and foot muscles have been produced until ninety to one hundred and twenty minutes after death. Some investigators report residual activity up to forty-eight hours postmortem. Idiomuscular contractions, those contractions produced by mechanically striking a muscle at right angles to its tissue, can bring about contraction of the whole muscle within one to two hours postmortem, but rarely within more than five hours. This test is complicated by the onset of rigor mortis. Excitability of face and hand muscles to electrical stimulation is usually lost by three hours after death. Pupillary reactions of miosis or mydriasis stimulated by pilocarpine and homatropin respectively are lost as early as three hours postmortem or as late as twenty hours postmortem. Ophthalmologists have recorded retinal changes of vascularization, color, and clarity, but only up to fifteen hours postmortem. Excitability of sweat glands has been measured using adrenaline as a stimulant and a dry starch-iodide layer on the skin as an indicator. Maximum response of sweat glands shows at sixty to ninety minutes after death. Some researchers have claimed to have observed responses up to thirty hours after death, but Schleyer (1963) expressed concern over the reproducibility of this work. In vitro

coagulation of blood has been used as a time of death assay by some. Coagulation will take place for as long as twelve hours postmortem, at the outside.

"When life has ended, dissolution begins, and the metabolic proceses of the living organism will either continue for a while and finally slow down and exhaust themselves, or they will be transformed, with new substances being produced. Actually this new chemical 'life' represents the earliest stages of 'decomposition' in its widest sense." Schleyer's remarks (1963:269) set well the stage for investigation of biochemical changes after death. The rates of these changes, which in many cases begin during the process of dying itself, are greatly influenced by factors such as environmental conditions, cause of death, duration of agony, antemortem health, diet and habits, and also by the metabolic activity of the site chosen for sampling. Yet within this diversity biochemical changes do appear to follow some general rules, and the results of these changes can often be measured exactly by chemical means. Many researchers are encouraged to try to correlate the time scale of these changes with the postmortem interval.

In serum, biochemical changes begin almost immediately after death, noticeable in many of the commonly measured laboratory parameters. Henry and

Smith (1980) reviewed the usefulness of these measurements in the estimation of the postmortem interval. Peripheral blood glucose falls rapidly as it is consumed by glycolysis; it may, however, demonstrate a brief rise centrally due to intrahepatic glycogenolysis. Lactic acid concentration rises in the first twenty-four hours. Intra and extracellular fluid compartments' concentration gradients equilibrate so that serum sodium levels fall while those of potassium rise rapidly. Tissue enzyme levels in serum also increase rapidly. Substances such as bilirubin, urea, creatinine, and some hormones which are relatively stable chemically and depend upon complex tissue metabolism for their generation or removal, tend to remain at constant levels for longer periods of time. Inorganic phosphate and the nitrogenous breakdown products of muscle and other proteins (amino acids, creatine, and ammonia) appear to be the most valuable constituents of serum for approximating the postmortem interval. Even these constituents have such varied rates of change from one individual to another that no precise estimation of the time of death can be made from the results of any single determination. The maximum postmortem interval predictable by serum chemistry levels according to this review is about thirty hours.

Henry and Smith (1980) consider cerebral spinal

fluid a "cleaner" body fluid for postmortem studies since it is more isolated from decomposing tissues than is serum, and in the absence of intracerebral hemorrhage spinal fluid is virtually acellular. Chemical changes in the cerebral spinal fluid tend to parallel those in serum, but the changes are slower in spinal fluid. Rises in potassium, lactate and inositol levels seem to be the most likely indicators of time of death in this fluid, but these changes have shown at best only statistical significance and are generally unreliable for a time estimation. Better predicting power can be gained using a battery of select serum and cerebral spinal fluid tests, but all-in-all these fluids prove to be very disappointing sources of information.

Henry and Smith (1980) regard intraocular humors as being the most promising sources of postmortem time information. Both vitreous and aqueous humors are relatively isolated from putrefaction tissue changes and are easily obtainable with minimum danger of contamination. Changes in concentration of lactate, pyruvate, ascorbate, nonprotein nitrogen, sodium, chloride, magnesium, phosphate, and bicarbonate have been too variable to be useful. Coe (1969) measured several chemical parameters in postmortem human vitreous humor up to 17 hours after death. He noted no reliable pattern of change in the concentrations of sodium,

chloride, carbonate, calcium or urea nitrogen. Dufour (1982) confirmed that there is no consistent change of postmortem calcium concentration with increasing time since death. Coe (1969) noted that postmortem glucose concentrations of vitreous humor tend to diminish with time, but the rate of fall is extremely erratic. He observed that while potassium levels rise arithmetically with respect to time after death, there is a marked individual variation in the rate of rise. He concluded that the measurement of potassium concentration in vitreous humor has limited value in determining the postmortem interval. Yet much investigation has been focused on postmortem vitreous potassium. Naumann (1959) demonstrated the first evidence of rising potassium levels in this fluid and suggested that the lysis of cell membranes in the vascular choroid was the source. Sturner and Gantner (1964) used this biochemical change as a means to estimate the time of death, claiming that the test was useful up to one hundred hours with a standard error of plus or minus 4.7 hours that did not increase with time. Henry and Smith (1980) report that several researchers have evaluated the relationship between postmortem vitreous humor potassium concentration and the postmortem interval with varied results...some claim even better correlation than Sturner and Gantner (1964), others insist that the test

is not usable, or demonstrates very poor confidence. There is a general agreement, however, that there is at first (six to twelve hours) a more rapid potassium concentration increase followed by a fairly linear rise from twenty-four to one hundred or even one hundred and twenty hours, reaching an eventual maximum of 25-40 mEq/L. Discrepancies could be related to differences in analytical techniques.

Other intraocular fluid studies have shown some promise. Van den Oever (1978) reported an ammonia concentration increase in vitreous humor very much like that of potasium. He also measured potassium levels in aqueous humor and found good time correlation; these levels are generally higher than those of vitreous humor, and their increase shows considerable temperature dependency. A novel study of the rate of change of a vitreous humor constituent level with time was presented by Adjutantis and Coutselinis (1974) relating the rise in vitreous magnesium with postmortem immersion time in sea water, sea water having a much higher initial concentration of magnesium. Equilibrium is reached by about twenty-four hours. This assay has a rather limited applicability of estimating relative order of deaths in the case of multiple victims of a sea disaster.

No sources to date mention gall bladder bile as a

potential fluid for a time of death assay. However, a large volume of literature has become available concerning the physiology of the gall bladder and the formation and composition of bile. Certain facts pertinent to this study have emerged from this literature.

Haslewood (1967) reports hepatic (fistula) bile protein concentrations of 0.2-1.2 g/100 ml which concentrate to 1.0-4.0 g/100 ml in the gall bladder. Strange (1984) claims that albumin is the predominant protein in bile, originating from the plasma pool, but present at lower concentrations than in plasma.

Several influences on the filling of the gall bladder by canicular flow from the hepatocytes have been recently investigated. According to Strange's review (1984), conjugated bile salts are the principle organic compounds in bile, and these bile salts stimulate canicular bile flow. Tavolini (1985) observed that bicarbonate is transported into bile, possibly at multiple sites within the biliary tree, and believes that the excretion of bicarbonate may be involved with the driving force for hormone-induced choleresis... accounting in part for the flow of bile associated with bile acid secretion. Anwer and Clayton (1985) noted that the extracellular calcium is essential for normal bile flow by way of maintaining the paracellular

permeability of hepatocytes necessary for bile acid excretion. Blitzer et al. (1986) postulated a new mechanism for bile acid transport across the hepatocyte that involves an energy-dependent sodium pump. The processes of bile formation and hepatic flow are also very drug sensitive as has been shown by Paumgartner (1979), Kern et al. (1982), and Okolicsanyi et al. (1986). These functions are depressed by contraceptive steroids like estrogen and lipid-lowering drugs like clofibrate; they are stimulated by a number of drugs, including phenobarbital, theophylline, glucagon, and insulin.

Banfield (1975) describes the concentrating process of bile as it is transformed from the dilute hepatic bile with the major cation being sodium and major anions consisting of chloride, bicarbonate, and bile salts to the more concentrated gall bladder bile with sodium still the major cation, but the only significant cation component being bile salt. This process is an activity of the gall bladder epithelial cells. Persson and Spring (1982) observed hydraulic water permeability in these cells. Ericson and Spring (1982) noted that there is a carrier-mediated sodium chloride uptake at the apical membrane of the gall bladder epithelial cell that is balanced by the active extrusion of sodium at the basolateral membrane. Weinman and Reuss (1982)

Weinman and Reuss (1982) determined that the sodium uptake at the apical membrane takes place in exchange for protons, explaining Bansfield's observation (1975) that the bile pH tends to drop 1-2 units during the concentration process. An interesting but unexplained observation of Svanvik and Jansson (1977) is that the concentrating process appears to stop temporarily in women undergoing C-section surgery; the gall bladder becomes distended and the gall bladder bile displays the same composition and concentrations as hepatic bile.

The periodic contractions and emptying of the gall bladder ultimately influence concentrations of bile components. According to Schoetz et al. (1981), at any given time the pressure on the gall bladder wall is maintained by passive elements comprised of fibroelastic tissue responses to filling and active elements comprised of tone and contractions of smooth muscle. Contractions of the smooth muscle, presumably leading to the emptying of the gall bladder, can be induced by pilocarpine, histamine and cholecystokinin or blocked by atropine. Sensitivity of the gall bladder muscle to chemical stimulus or supression is confirmed in work by Everson et al (1983), Mesgarzadeh et al. (1983), Davison and Shaffer (1986), Fisher et al. (1986), and Gullo (1986). Gall bladder emptying in females apparently is also sensitive to normal hormonal balance, as evidenced

by work of Everson et al (1982) who demonstrated that both the frequency and efficiency of gall bladder emptying are diminished in normal pregnancy. Pomeranz et al. (1983) tested the influence of pancreatic polypeptide and motilin on isolated strips of gall bladder smooth muscle and found no response; they concluded that the in vivo activity of these compounds on gall bladder contraction must occur at a remote site rather than influencing the muscle directly. Work by Takahashi et al. (1982) with dogs revealed gall bladder contractile activity independent of the response to feeding. Peeters et al. (1980) had observed an association between bile acid output into the duodenum and the periodic waves of upper intestinal motor activity. Contractions are stimulated by what they referred to as the "interdigestive migrating motor complex". Itoh et al. (1982) noted that interdigestive contraction activity in dogs do in fact influence the concentrating of bilirubin in the gall bladder bile.

The unique sum of these varied conditions and stimuli influences the concentration of gall bladder bile components at any given moment. Each step in the production of gall bladder bile, hepatic bile formation and flow, gall bladder concentration, and gall bladder emptying, has some energy-dependent component and should come to a complete halt following the event of death.

The action of some bacteria could influence the rate of decomposition of gall bladder bile. Munro and Sorell (1986) stated that bile in the absence of disease in the biliary tract is either sterile or contains few bacteria. Any bacteria present in bile are the same as those found in the gastrointestinal tract. Mechanisms of colonization are uncertain, but bacteria are believed to either ascend from the duodenum or are derived from portal or systemic circulation. Interestingly, Wilson et al. (1972) reported 49% positive postmortem liver cultures in autopsies with no apparent infections.

In the last 10-15 years the literature has opened up on applications of high pressure liquid chromatography (also called "high-performance" or even "high-speed", terms emphasizing different characteristics of the same methodology). Several authors (Done et al., 1974; Dixon et al., 1976; Lawrence, 1981; Soldin, 1982; and Parris, 1984) have described its usefulness in pharmaceutical analysis, clinical testing of biological fluids and tissues, environmental monitoring, food analysis, analysis of agricultural chemicals and analysis of petroleum and related products. There has been considerable interest in its application to the analysis of bile acids and bilirubin in both animal and human bile. Recent researchers in this area have been Onishi et al. (1980),

Takeuchi et al. (1983), Carducci et al. (1985), Hayashi et al. (1985), Swobodnik et al. (1985), and Spivak and Yuey (1986). Advantages of using ion-exchange high pressure liquid chromatography on certain biological components were described by Brown (1973) and Rosset et al. (1979). Generally this methodology applies well if the components are water soluble and ionized or are easily ionizable by modifying pH. One can expect good separation of polar and ionic compounds. Ion-exchange columns adapt well to high pressure; they are quite stable, do not degrade easily, and usually offer low resistance to fluid flow.

The specific method used for cations in this study was described by Wetzel and Woodruff (1978) and was adapted from work by Bouyoucos (1977). The hydroxides of the eluted cations are put through a second column that converts them to more highly dissociable chlorides before they are subjected to a conductivity detector. This method assures a better linear quantitative response for more weakly dissociating cations like ammonia and small amines. The anion assay is presented in the general instructions of the DIONEX manual (1978). The manual describes the use of a dilute borate eluent to extend retention times, thus producing better separation of simple short-chain carboxylic acids without chloride interference.

CHAPTER III

MATERIALS AND METHOD

Equipment used for this study included an MSE Centrifuge, Model "Super-minor". Cation and anion assays were run using a DIONEX Model 10 Ion Chromatograph with DIONEX cation separator column HPIC-CS1 and suppression column CSC-1, and anion separator column HPIC ASC-1 and suppression column ASC-1. Flame photometry was run on an IL Model 143.

Deionized water was twice distilled in glass before being used for all reagents and standard solutions. Water for the eluants and standards used in the anion assay was degassed by boiling for five minutes and protected from ambient CO2 by a bubble trap containing 1 N NaOH.

Reagent grade chemicals were used for all extracting and eluting preparations. Sodium borate (Na2B407.10 H20), sodium bicarbonate, sodium carbonate, concentrated hydrochloric acid and sodium hydroxide pellets were all from Baker Chemical Company. Trichloroacetic acid solution (6.25%,W/V) was from Sigma Chemical Company.

Chemicals used for standards included reagent grade ammonium chloride, potassium chloride, sodium chloride, sodium acetate, sodium citrate (dibasic), sodium acetate, sodium phosphate (dibasic anhydrous), and sodium sulfate by Baker. Reagent grade sodium nitrate was from Mallinckrodt and reagent grade sodium formate, from Fisher. Best possible grade of monomethylamine, dimethylamine, trimethylamine, ethylamine, pyruvic acid (sodium salt), DL-glyceric acid (hemi-calcium salt), cholic acid (sodium salt), and α-ketoglutaric acid (free acid) were obtained from Sigma Chemical Company and were used with no further purification.

B-D sterile disposable syringes and needles were used to extract the bile samples.

Dr. Robert Lawrence of Delta Medical Laboratories provided the human gall bladders. Each gall bladder was received directly from autopsy under refrigeration (0-5° C) and was processed immediately. The bladder was placed fundus downward in a glass screw-top container (250 ml capacity) and its weight was recorded (See Table 1.). An initial sample of bile (1-2cc) was withdrawn from the body of the bladder, just below the neck, with a 5cc syringe equipped with a 17-gauge needle. The container was then closed and the bladder was protected from light by placing the container inside a brown bag. It was left at room temperature (23±3° C) until the next

sampling. Bile was extracted and ambient temperature was recorded (Table 1.) at 24±1/2 hour intervals for seven consecutive days. Samples had to be extracted from successively lower sites on the body of the gall bladder as the incubation progressed. The portions of bile were frozen immediately. Bile from the first gall bladder received was removed in only two large portions, one at the initial time and the remainder after seven days' incubation, providing a quantity of material with which to establish methods and extremes of assay. A total of twelve specimens were received and incubated across a period of five months.

When all the samples were collected, they were thawed in lots of six to eight and 1/2cc of each was added rapidly with vortex mixing to 2cc of 6.25% trichloroacetic acid to remove protein. After 30 seconds of vortexing, each sample was centrifuged for 15 minutes at 10,000 rpm, and the clear supernatant was transferred to a polypropylene specimen container and refrozen.

Cation assays on all samples taken from one gall bladder were run on a single day. When all cation assays were completed, the anion assays were run in a like manner. Peak identification was made by comparison of retention times with those of standards (Table 2.).

Quantitation was achieved by using external standards and comparing the products of net heights times the widths at the half-heights. Thawed trichloroacetic acid extracted samples were diluted 8X with water, yielding a net 40X dilution before direct injection into the chromatograph. Injection volume was 100 microliters.

The standard for quantitation of the cation asay contained 5 ppm sodium, 5 ppm ammonium ion, and 10 ppm potassium, dissolved in 0.001 M HCl to prevent volatilization of ammonia. Aliquotes of this standard were frozen and stored beside the extracts of bile. Supplementary cation standards of 30 ppm each of monomethylamine, dimethylamine and trimethylamine, as well as 50 ppm ethylamine were also dissolved in 0.001 M HCl. Eluant for the cation assay was 0.005 M HCl. Sodium and potassium levels of the initial-time samples of each gall bladder were assayed using the flame photometer for comparison (Table 3.).

The anion quantitation standard contained 10 ppm acetate, 5 ppm formate, and 10 ppm chloride. Supplementary standards contained 20 ppm nitrate, 100 ppm phosphate, 100 ppm sulfate, 50 ppm oxalate, 50 ppm citrate, 10 ppm, pyruvate, 100 ppm glycerate, 100 ppm cholate, and 100 ppm, α-ketoglutarate. The anion standards were all prepared in degassed water, and fresh preparations only were used for quantitation.

The anion eluant was 0.005 M sodium borate. A second eluant of 0.0024 M NaHCO3, 0.0019 M Na2CO3, and 0.001 M Na2B4O7 was used to clear the separating column of nitrate, phosphate, sulfate, and trichloroacetate between assays. The pump was maintained at 30% delivery giving a measured flow rate of 2.7±0.1 ml per minute. Chart speed was 0.2 inch per minute; recorder was set at full scale equal to 10 mho (reciprocal ohms), except during the quantitation of sodium which required 100 mho full scale.

CHAPTER IV

RESULTS

Figures 1 through 11 show the time-sequenced chromatograms of bile from the second through the twelfth gall bladder respectively. An event marker indicates injection of sample for each chromatogram for days 0 through 7 of incubation. The peaks were designated A through E in order of appearance in each chromatogram; A corresponds to sodium, B to ammonium ion, and C to potassium. The identities of peaks D and E are less certain. D appears at a position shared by dimethylamine and ethylamine. These two amines have essentially the same standard conductance. Peak E corresponds to no known standard; this peak was assigned the same standard conductance as peak D.

Computed concentrations of ammonium ion and potassium in parts per million are given in Table 4; estimated concentrations of peaks D and E are listed in Table 5. The ammonium ion concentrations for each gall bladders appear to increase linearly; this data was submitted to regression analysis and the slopes of these concentration changes vary from approximately 2 ppm per

day to greater than 60 ppm per day. Initial ammonium ion levels range from 12 to 96 ppm. Equality of the slopes was tested by the method described by Mendenhall (1987) and it was found that there are no significant differences between the slopes of gall bladders numbered 2, 4, 5, and 6. Another cluster of related slopes includes those of gall bladders numbered 8, 9, 10, 11, amd 12. The slope of gall bladder number 3 is significantly different from all others. Ammonium ion concentration changes in gall blader number 7 show poor linear correlation with incubation time. The antemortem health and habits as well as the actual causes of death which may relate to the differences between clusters of slopes are unknown in this study.

Potassium concentration remains essentially constant across the seven day incubation of each gall bladder; only three of the eleven gall bladders show a significant linear correlation between potassium level and incubation time, and the slopes of these three are very small...only 2-5 ppm per day Peaks D and E do vary with incubation, but follow no consistent pattern of concentration change.

Sodium, which requires a rerun at different full scale recorder setting for quantitation, was measured on day-one samples from each gall bladder, and the seventh day of the first specimen and sixth and seventh days of

the fourth specimen. Data from these samples indicate that no change of sodium concentration occurs with increased incubation time. No further work was done on this constituent.

Figures 12 through 22 contain the anion chromatograms on the second through the twelfth gall bladders. Again the peaks were designated A through D by order of appearance. Peak A corresponds to acetate. Peak B, not identified, appears between A and C and is not well resolved from the acetate peak A preceding it. Peak D was not identified, and occurs with such irregularity that quantitation was not attempted. Estimated concentrations of peaks A and B are included in Table 6; peak C , in Table 7. There appears to be no pattern of concentration change among these small anions that could be used to predict the postmortem interval.

CHAPTER V

DISCUSSION

From the ions studied in this work, the ammonium ion shows the most consistent change with respect to incubation time. The linear increase demonstrated in individual gall bladders leads one to believe that a useful correlation can be found. The wide range of slopes must be confronted and can be, by a couple of different approaches.

One way to deal with this diversity would be to look at the initial time intercepts: do the different plots come to a common focus? If so, one could incubate any unknown postmortem gall bladder, taking daily samples long enough to determine a slope, and estimate time of death by extrapolating back to reach a common starting level. Although the range of initial ammonium ion levels of these eleven gall bladers is from 12 to 96 ppm, the majority of the starting concentrations do fall within a rather small cluster at 40 ppm or less. This focusing of initial concentrations should be even sharper when data is obtained relating to actual times of death instead of to the starting times of an <u>in vitro</u>

incubation.

Another way to evaluate the ammonium ion concentration relative to time is to compare that concentration to the simultaneous concentration of another bile constituent and see if the relationship between the two follows a meaningful pattern with increasing time. The relationship between ammonium and potassium ion concentrations determined in this study provides a clear visualization of increase with respect to incubation time. The ratios of these concentrations were computed to express this observation mathematically (Table 8.). Figure 23 shows the distribution of these ratios as a function of time. In four of the eleven cases the ratios equaled or exceeded unity by day four; in five of the eleven, by day five; and in six of the eleven, by day six. Not one of the gall bladders presented an ammonium ion to potassium ratio of unity before day four. Therefore, a ratio of unity or greater between these to components appears to indicate a postmortem interval of at least four days, and about half of the individuals do show a ratio of this size. While these treatments of the ammonium ion concentration, extrapolation of a slope back to an initial level or comparison of the ratio of concentration of ammonium ion to that of potassium, have limited predicting value when used separately, they

could be incorporated in with other tests and observations, providing the investigator with at least one more clue in an area where clues are few.

A variety of concentration changes, either positive or negative, are apparent with acetate, formate, and at least one other major constituent so far unidentified in the anion assay. At this time there seems to be no useful time correlation among these patterns. Further studies may reveal more possibilities in this area.

The in vitro model for a new biochemical assay from postmortem bile has revealed one reasonable candidate for correlation with time of death...the ammonium ion concentration, either with consecutive samples taken to determine slope or with potassium levels to compare ratios. Of these two treatments, the latter would be the simplest to employ, and in that respect, may be the more appealing to investigate further for application to time of death determinations. The determination of slope, on the other hand, offers the hope of better information for the effort.

CHAPTER VI

SUGGESTIONS FOR FURTHER WORK

While results with anions were disappointing in this study, there are possibilities for improvement in the methods used on these constituents. The trichloroacetic acid extraction appeared to work very well for ammonium ion concentration, but it is probably not the best deproteinizing method for quantitation of anions. Small anions might be more stable in a solution that is neutral or alkaline. The clear trichloroacetate extract of these samples could be neutralized immediately after centrifugation, but one still must deal with high residual levels of trichloroacetate itself...a considerable nuisance on the anion column. An alternate deproteinizing agent should be sought.

Resolution of the monofunctional aliphatic carboxylic acids is rather difficult to attain. These anions are very weakly retained on ion exchange resins, and therfore they tend to elute too quickly or with overlapping peaks, even in the presence of a very weak eluant. Perhaps the borate concentration could be reduced even further without loss of stability; or maybe

an altogether different eluant could be found to work better. Altering other parameters of the anion assay such as increasing column length, varying pH, or raising or lowering temperature might also improve resolution.

More studies could be done using the in vitro incubation of gall bladders while correlating other factors that may influence the rate of increase of ammonium ion concentration. A simple protein assay on the initial bile sample followed by incubation of the gall bladder with daily cation assays would demonstrate any correlation between the rate of increase of ammonium ion and the amount of bile protein available for putrefaction. Similarily, initial amino acid concentrations...especially glutamine....might provide valuable insights into the cause of such a diversity of rates. Temperature dependency studies might extend the applicability of this test.

The most reasonable approach for further work at this time might be to bypass totally the in vitro model and compare ammonium ion levels of individual bile samples taken directly from gall bladders at autopsy of cases where time of death is either being established by other means or has already been documented. This preliminary work was designed with the goal in mind of moving on to exactly this approach. Bile is usually easy to extract during autopsy and in fact is withdrawn

for other assays, especially for the search for toxic substances or their metabolites. The trichloroacetic acid extraction for sample preparation is simple and is already in use in medico-legal laboratories for serum and whole blood alcohol assays. This treatment provides a sample that is relatively stable for easy transport and storage. The cation chromatogram itself is short and easy to produce once the system is established; numerous consecutive assays can be run with no between-run column preparation. A large data base could be compiled from cooperative laboratories representing a wide geographic area. This data could verify the usefulness of postmortem ammonium ion concentration in bile as an indicator of time of death.

CHAPTER VII

SUMMARY

The possibility of using the measurement of concentrations of small cations or anions in gall bladder bile as evidence for determining the postmortem interval was investigated. Human gall bladders from autopsy were incubated for seven days at room temperature. Daily samples of bile from these gall bladders were subjected to high pressure liquid chromatography by ion exchange. Peaks were identified and quantified for sodium, ammonium, and potassium ions on the cation column and for acetate and formate on the anion column. Of these ions only ammonium ion displayed a regular change of concentration with respect to incubation time, increasing linearly in each individual gall bladder, with varied rates of increase. While any single measurement of ammonium ion concentration in postmortem bile cannot alone determine the time of death, an understanding of cause for the variety of rates of change may reveal a useful corellation. Further study on the relationship of the ammonium ion concentration in bile to the postmortem interval seems justified.

Table 1. Gall Bladder Incubation Statistics: Mass at Starting Time and Daily Ambient Temperature Readings

Gall Bladder Number	Mass grams	Day: 0	Ambient Temperature in Degrees Celsius 1	2	3	4	5	6	7
1	72	25.5	25.1	24.9	25.2	25.4	24.8	26.0	25.9
2	69	21.8	22.7	22.4	23.6	22.9	25.8	25.7	24.9
3	141	24.6	22.8	22.3	22.1	23.5	22.9	24.5	24.8
4	70	23.5	23.0	23.5	24.3	25.4	23.6	23.6	21.2
5	117	23.5	23.0	23.5	24.3	25.4	23.6	23.6	21.2
6	60	22.1	23.5	23.0	24.5	23.9	26.1	24.7	25.6
7	153	22.3	23.2	21.6	23.0	22.4	22.1	21.9	22.0
8	82	21.6	23.0	22.4	22.1	21.9	22.0	21.3	21.1
9	112	19.8	21.0	20.7	20.2	20.1	20.4	20.3	21.9
10	104	20.1	21.3	23.7	----	20.4	20.0	21.33	20.2
11	81	21.3	20.1	20.8	20.7	20.4	20.2	21.1	21.7
12	94	22.7	23.2	23.0	22.0	20.8	21.3	21.1	21.7

Table 2. Retention Times of Cation and Anion Standards and Bile Samples on DIONEX Chromatograph Columns.

Cation	Time minutes	Anion	Time minutes
Volume front	1.9	Volume front	1.8
Sodium	3.6	Acetate	3.0
Ammonium	4.2	Formate	3.6
Monomethylamine	4.9	Pyruvate	4.2
Potassium	5.3	Chloride	7.5
Dimethylamine	7.0	Bile Peak A	3.0
Ethylamine	7.0	Bile Peak B	3.2
Trimethylamine	14.1	Bile Peak C	3.6
Bile Peak A	3.6	Bile Peak D	3.9
Bile Peak B	4.2		
Bile Peak C	5.3		
Bile Peak D	7.0		
Bile Peak E	7.7		

Table 3. Sodium and Potassium Concentrations in Bile Samples by DIONEX Chromatograph Compared to Flame Photometry.

Gall Bladder Number	Sodium			Potassium		
	mEq/L Flame	ppmX100 DIONEX	mEq/L DIONEX	mEq/L Flame	ppmX100 DIONEX	mEq/L DIONEX
1	36	8.8	38	7.6	2.8	7.2
2	49	12.0	52	7.5	3.1	7.9
3	31	7.6	33	3.0	1.2	3.2
4	38	9.2	40	5.5	2.2	5.5
5	28	6.4	28	4.1	1.6	4.2
6	24	5.2	23	6.8	2.7	6.9
7	32	7.6	33	2.9	1.1	2.9
8	40	8.8	38	3.2	1.2	3.0
9	34	6.8	30	6.4	2.4	6.0
10	31	7.2	31	4.7	1.9	4.9
11	29	6.4	28	5.7	2.1	5.3
12	32	7.2	31	6.4	2.4	6.2

Table 4. Ammonium and Potassium Ion Concentrations in Daily Bile Samples from Incubated Human Gall Bladders.

Gall Bladder Number	Day: 0	1	2	3	4	5	6	7	Slope	r
	Ammonium Ion, ppm X 100									
1	0.4	---	---	---	---	---	---	5.5		
2	0.4	0.4	1.2	2.2	2.8	3.3	3.6	4.0	.57	.98
3	0.3	0.4	0.6	0.8	1.1	1.6	1.9	2.3	.30	.98
4	0.2	0.5	1.2	1.8	2.6	2.9	4.0	4.0	.57	.99
5	0.3	0.3	0.6	1.1	2.4	3.2	3.6	3.9	.61	.97
6	0.4	0.5	1.2	2.2	2.6	2.9	3.5	3.6	.50	.98
7	0.3	0.2	0.2	0.2	0.2	0.3	0.3	0.4	.02	.66
8	0.1	0.1	0.3	0.7	0.8	0.8	0.8	0.8	.12	.91
9	0.5	0.5	0.6	0.7	0.9	1.1	1.2	1.4	.14	.98
10	1.0	1.3	1.6	---	2.0	1.9	2.1	2.2	.17	.96
11	0.8	0.8	1.0	1.6	1.7	1.8	1.9	1.9	.19	.93
12	0.3	0.2	0.4	0.7	0.9	1.1	1.2	1.2	.16	.98
x	0.4	0.5	0.8	1.2	1.6	1.8	2.0	2.2		
s	0.3	0.3	0.4	0.7	0.8	1.0	1.2	1.3		
v	62	74	56	59	55	56	59	59		
	Potassium Ion, ppm X 100									
1	2.8	---	---	---	---	---	---	---		
2	2.8	2.3	2.3	2.6	2.6	2.6	2.6	2.7	.03	.33
3	1.2	1.3	1.3	1.2	1.4	1.5	1.4	1.5	.04	.86
4	2.0	2.1	2.2	2.2	2.2	2.0	2.3	2.2	.03	.63
5	1.6	1.8	2.0	2.0	2.0	2.0	2.0	2.1	.05	.83
6	2.7	2.9	2.8	2.9	2.8	3.0	2.8	2.8	.02	.48
7	1.1	1.2	1.1	1.1	1.1	1.1	1.1	1.1	-.01	-.62
8	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	.00	-.25
9	2.4	2.4	2.4	2.5	2.4	2.6	2.5	2.4	.01	.54
10	1.9	1.9	2.0	---	2.0	1.9	2.0	2.0	.01	.39
11	2.1	2.0	2.1	2.2	2.2	2.2	2.2	2.2	.02	.86
12	2.4	2.1	2.4	2.4	2.3	2.3	2.4	2.2	-.01	-.17
x	2.0	1.9	2.0	2.0	2.0	2.0	2.0	2.0		
s	0.6	0.5	0.6	0.6	0.6	0.6	0.6	0.6		
v	31	28	28	32	28	30	29	28		

Table 5. Concentrations of Cations D and E in Daily Bile Samples from Incubated Human Gall Bladders.

Gall Bladder Number	Cation D, ppm X 100 Day: 0	1	2	3	4	5	6	7
1	0.8	---	---	---	---	---	---	5.1
2	0.8	0.8	0.8	1.5	1.6	1.8	2.2	2.7
3	0.8	0.8	0.8	0.8	0.9	1.1	1.1.	1.2
4	---	---	*	*	*	1.4	3.0	3.6
5	0.8	0.8	0.8	0.8	0.9	1.7	3.1	3.7
6	*	*	1.8	2.2	3.3	5.5	6.6	8.4
7	*	*	0.8	0.8	1.2	2.2	2.8	3.8
8	---	---	---	0.8	0.8	0.8	0.8	0.8
9	---	---	0.8	0.8	0.9	1.4	1.8	2.4
10	1.1	1.4	1.8	---	2.4	2.5	2.6	2.9
11	0.8	0.8	0.8	0.9	1.7	3.0	3.6	4.1
12	---	---	---	---	---	---	---	---
Gall Bladder Number	Cation E, ppm X 100 Day: 0	1	2	3	4	5	6	7
1	---	---	---	---	---	---	---	---
2	---	---	---	---	---	---	---	---
3	---	---	---	---	---	---	---	---
4	0.8	0.8	0.8	0.8	0.8	0.8	---	---
5	---	---	---	---	---	---	---	---
6	1.1	1.0	0.8	0.8	0.8	0.8	0.8	0.8
7	0.9	0.8	0.8	0.8	0.8	0.8	0.8	0.8
8	---	---	---	---	---	---	---	---
9	1.3	1.3	1.1	1.1	1.0	0.8	0.8	0.8
10	---	---	---	---	---	---	---	---
11	---	---	---	0.8	1.3	1.4	1.0	0.8
12	---	0.8	0.8	0.8	0.8	1.4	1.8	2.6

*Present, but not separated well enough to quantify.

Table 6. Concentrations of Acetate and Acetate Plus Unidentified Anion B in Daily Pile Samples from Incubated Human Gall Bladders.

Gall Bladder Number	Day: 0	1	2	3	4	5	6	7
	Acetate Ion, ppm X 100							
1	5.2	---	---	---	---	---	---	---
2	4.8	5.6	8.4	7.6	*	*	*	*
3	3.2	4.0	4.0	4.0	*	*	*	*
4	4.8	4.0	*	*	*	*	*	*
5	4.4	5.2	5.6	*	*	*	*	*
6	5.1	6.8	*	*	*	*	*	*
7	1.0	1.8	*	*	*	*	*	*
8	0.4	1.2	1.2	*	*	*	*	*
9	3.2	3.2	3.4	4.0	*	*	*	*
10	2.0	1.2	0.8	---	*	*	*	*
11	2.2	2.6	3.2	4.0	*	*	*	*
12	2.2	3.8	5.0	5.6	5.4	5.0	5.2	5.2
Gall Bladder Number	Day: 0	1	2	3	4	5	6	7
	Acetate Plus Anion B, ppm X 100							
1	---	---	---	---	---	---	---	11.2
2	---	---	---	---	9.6	10.4	10.0	11.6
3	---	---	---	---	6.0	7.2	5.2	7.8
4	---	---	4.0	6.0	8.0	9.2	12.8	14.0
5	---	---	---	8.0	10.0	11.2	12.0	15.2
6	---	---	5.2	7.2	6.0	8.8	10.8	10.8
7	---	---	1.8	1.0	1.5	1.2	1.2	0.9
8	---	---	---	1.2	1.2	0.8	0.8	0.8
9	---	---	---	---	3.8	3.2	3.2	4.4
10	3.2	2.4	2.8	---	3.2	2.8	2.8	---
11	---	---	---	---	3.4	2.8	2.4	2.0
12	---	---	---	---	---	---	---	---

*Cannot be quantified separately from anion B.

Table 7. Concentrations of Formate Ion in Daily Bile Samples from Incubated Human Gall Bladders.

Gall Bladder Number	Formate ion, ppm X 100							
	Day: 0	1	2	3	4	5	6	7
1	.08	---	---	---	---	---	---	.32
2	.08	.08	.32	.36	.40	.76	.76	.40
3	.08	.08	.20	.32	.24	.32	.20	.24
4	.08	.20	.48	.52	.48	.52	.96	.96
5	---	.08	.44	.68	.64	.60	.88	1.0
6	.08	.16	.96	1.0	1.1	1.4	.96	.64
7	.08	.08	.16	.16	.16	.08	.08	.08
8	---	---	.08	.08	.12	.12	.12	.12
9	---	---	.08	.24	.38	.40	.32	.24
10	.24	---	---	---	---	---	---	---
11	---	---	.56	1.2	1.1	1.1	1.1	1.1
12	---	---	.22	.46	.50	---	.38	.38

Table 8. Ratios of Ammonium and Potassium Ion Concentrations in Daily Bile Samples from Incubated Human Gall Bladders.

Gall Bladder Number	Day: 0	1	2	3	4	5	6	7	Slope	r
					Ratio					
1	.14	---	---	---	---	---	---	.82		
2	.16	.19	.50	.86	1.08	1.26	1.38	1.48	.21	.98
3	.23	.28	.42	.67	.80	1.06	1.31	1.54	.19	.99
4	.12	.23	.56	.83	1.14	1.41	1.75	1.82	.27	.99
5	.17	.15	.28	.54	1.22	1.60	1.82	1.85	.29	.96
6	.15	.18	.45	.78	.93	.97	1.23	1.25	.17	.98
7	.25	.20	.22	.18	.21	.26	.29	.37	.02	.66
8	.10	.10	.23	.58	.66	.69	.69	.69	.08	.91
9	.22	.21	.25	.29	.37	.42	.48	.59	.05	.97
10	.50	.67	.78	---	1.00	1.02	1.06	1.08	.08	.96
11	.40	.40	.45	.76	.80	.82	.85	.87	.08	.92
12	.11	.11	.18	.29	.38	.48	.51	.56	.07	.99
x	.21	.25	.40	.58	.78	.91	1.03	1.16		
s	.12	.16	.18	.24	.34	.42	.51	.54		
v	57	65	45	41	44	46	50	47		

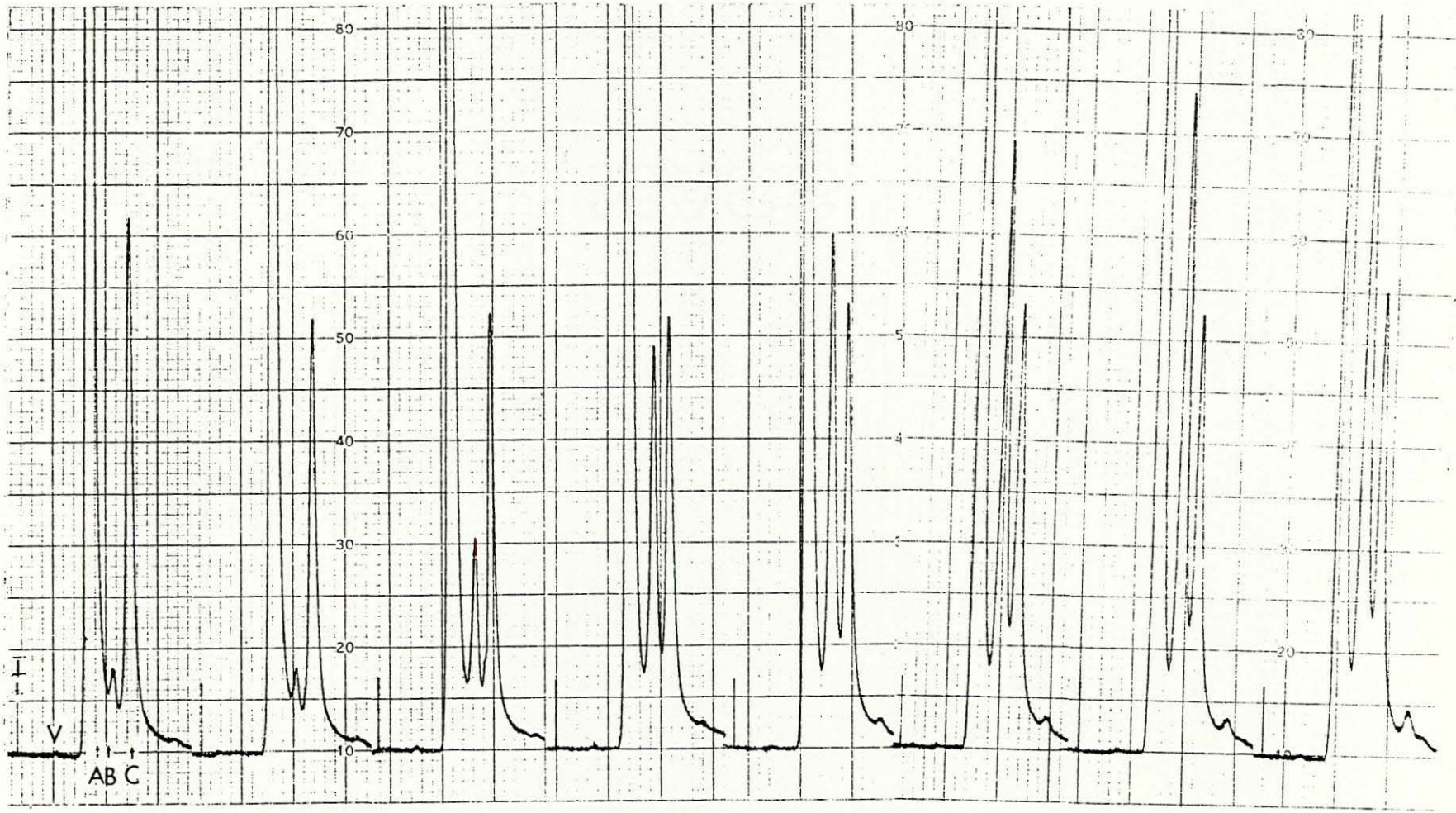

Figure 1. Cation Assays on Bile from Gall Bladder Number 2, Days 0 through 7 of Room Temperature Incubation. An event marker indicates sample injection g(I) beginning each chromatogram. Volume front (V) may be visible. Peaks in order of appearance are A, sodium (off scale); B, ammonium; and C, potassium. Small peaks following potassium are unidentified.

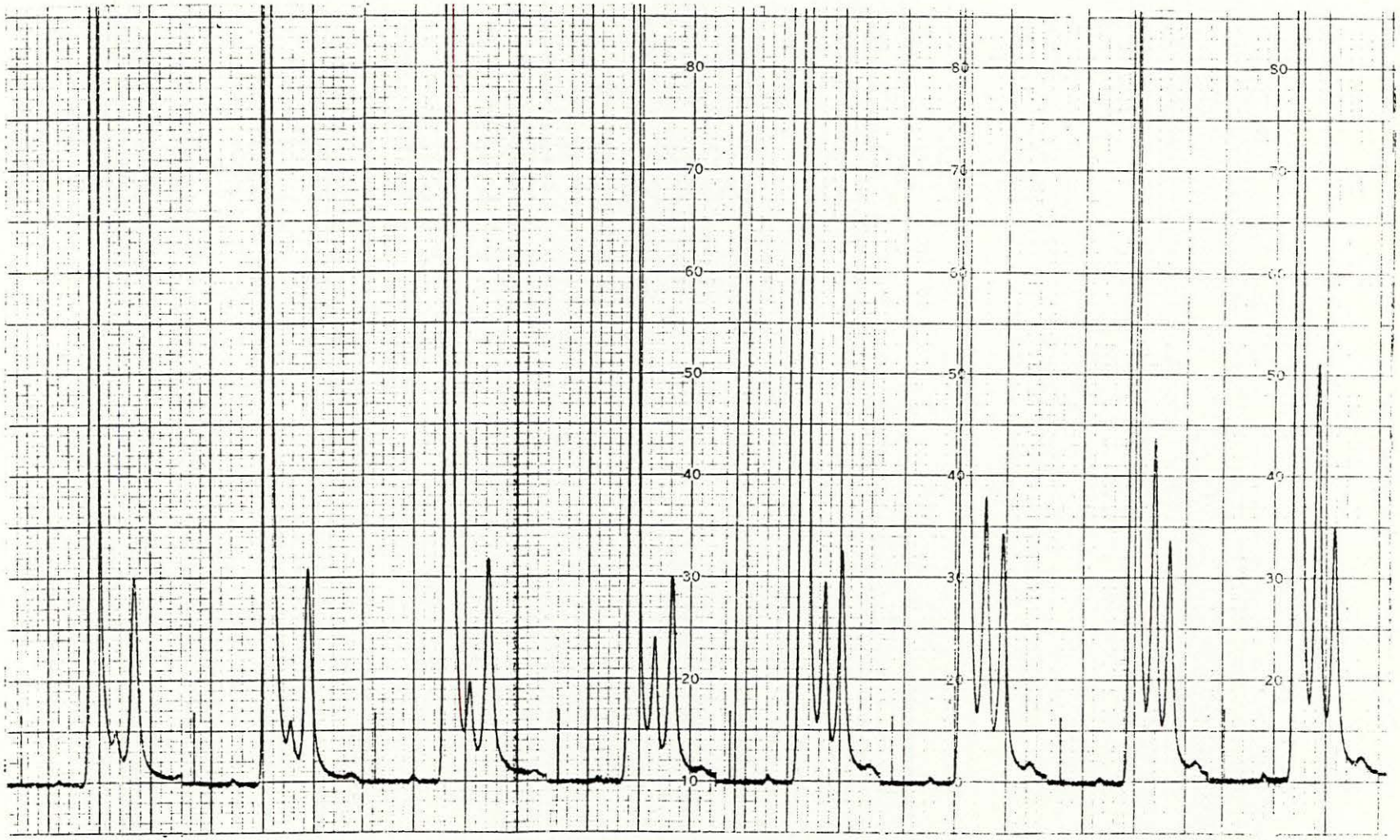

Figure 2. Cation Assays on Bile from Gall Bladder Number 3, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 1.

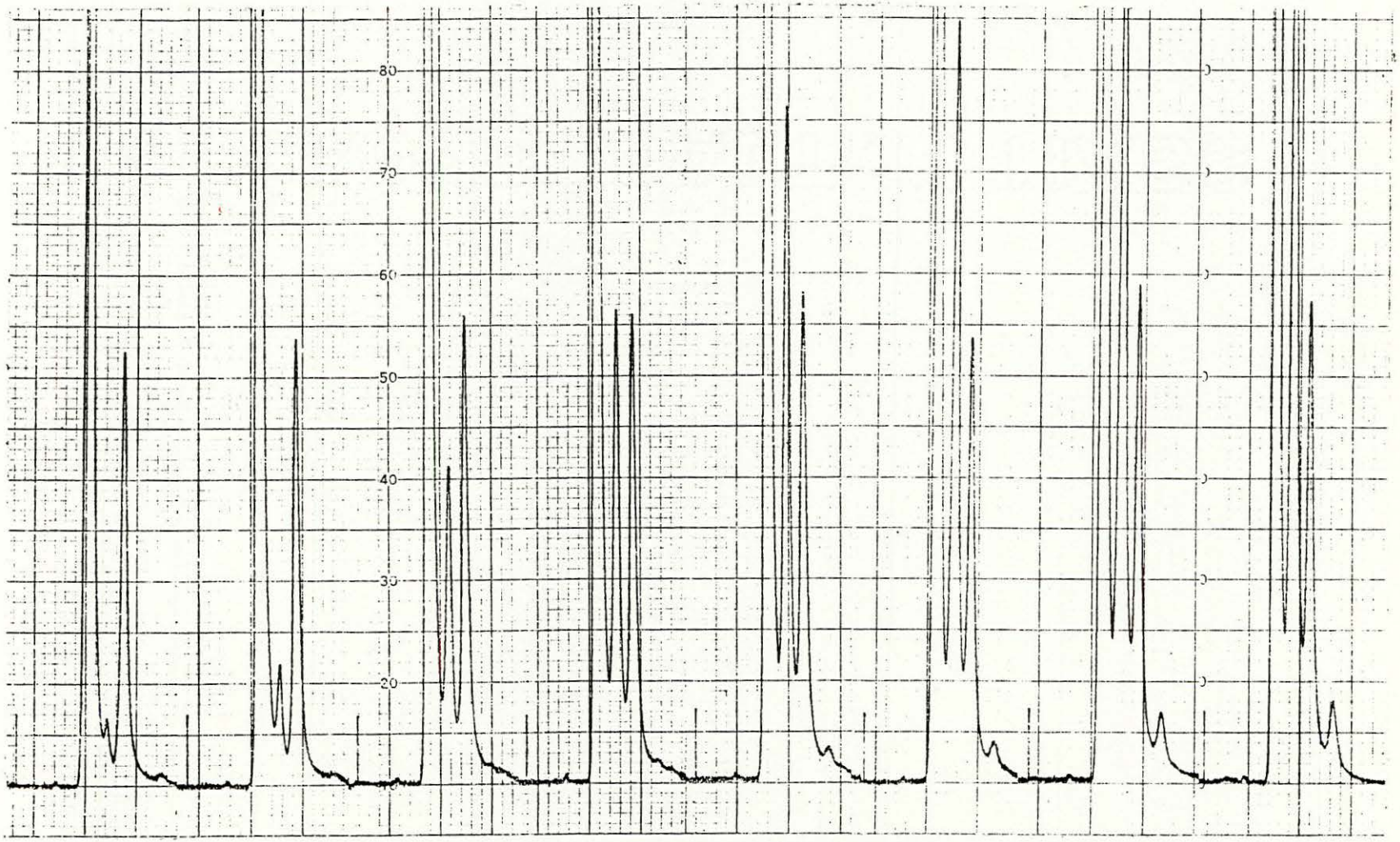

Figure 3. Cation Assays on Bile from Gall Bladder Number 4, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 1.

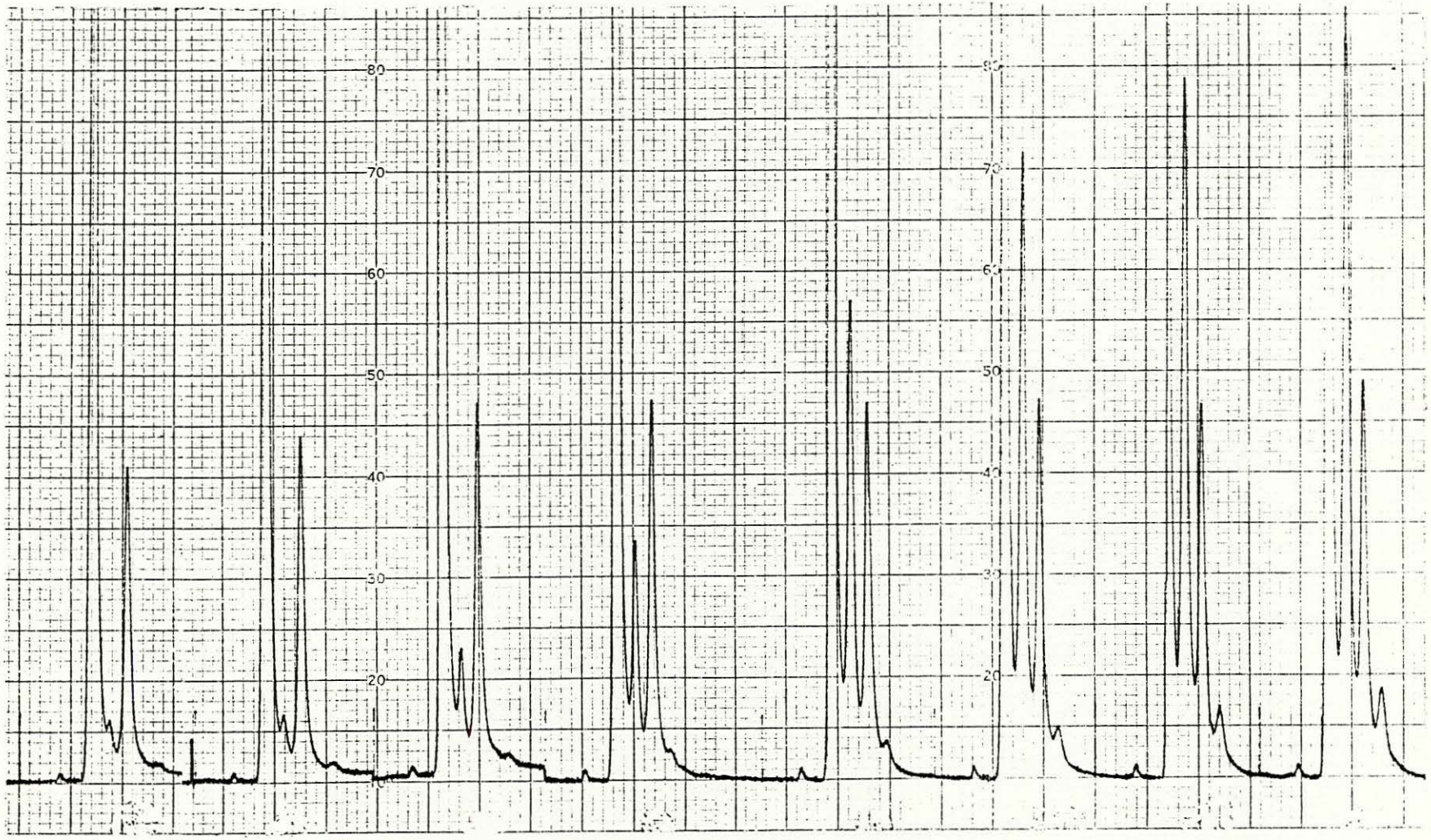

Table 4. Cation Assays on Bile from Gall Bladder Number 5, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 1.

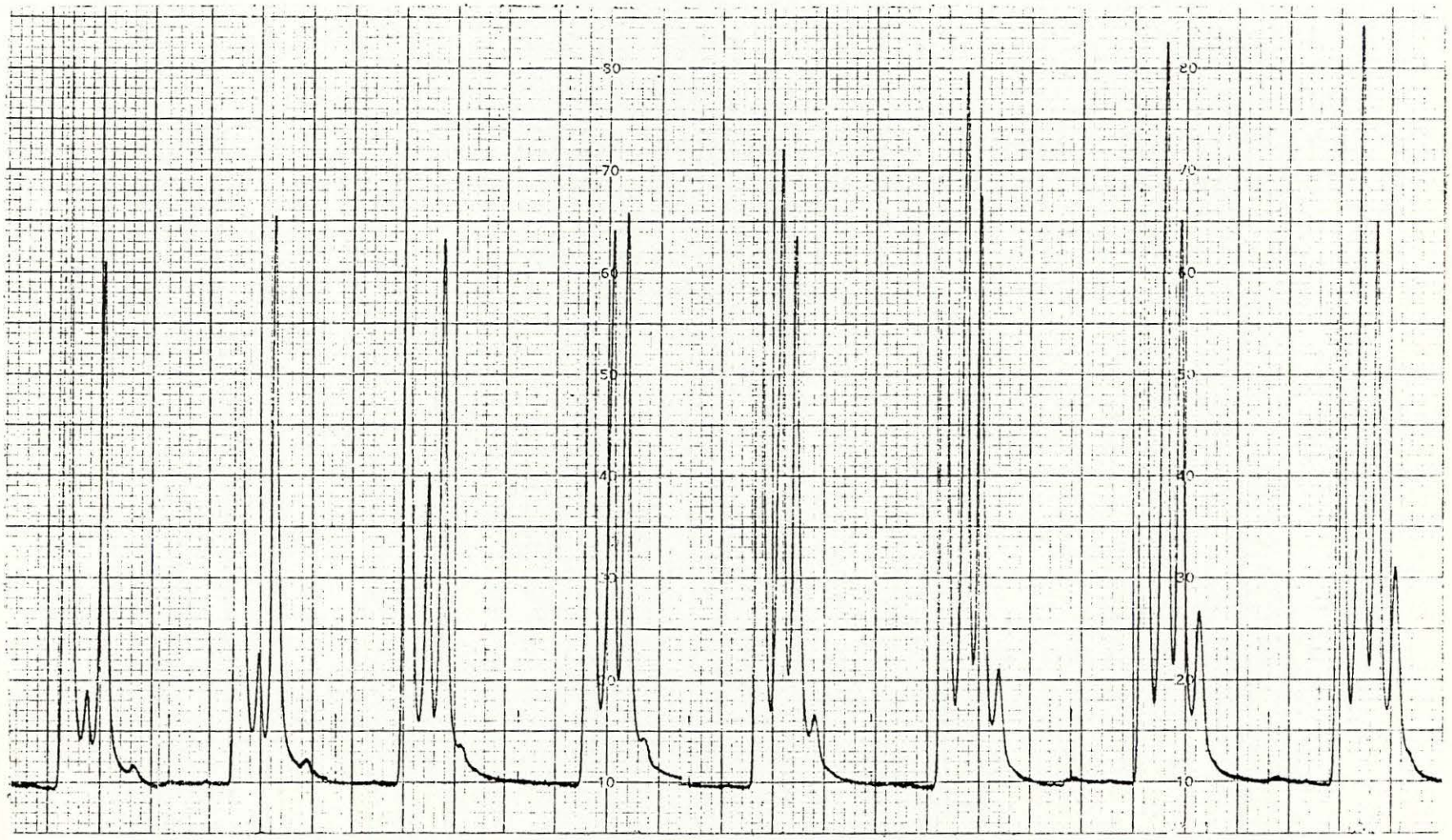

Figure 5. Cation Assays on Bile from Gall Bladder Number 6, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 1.

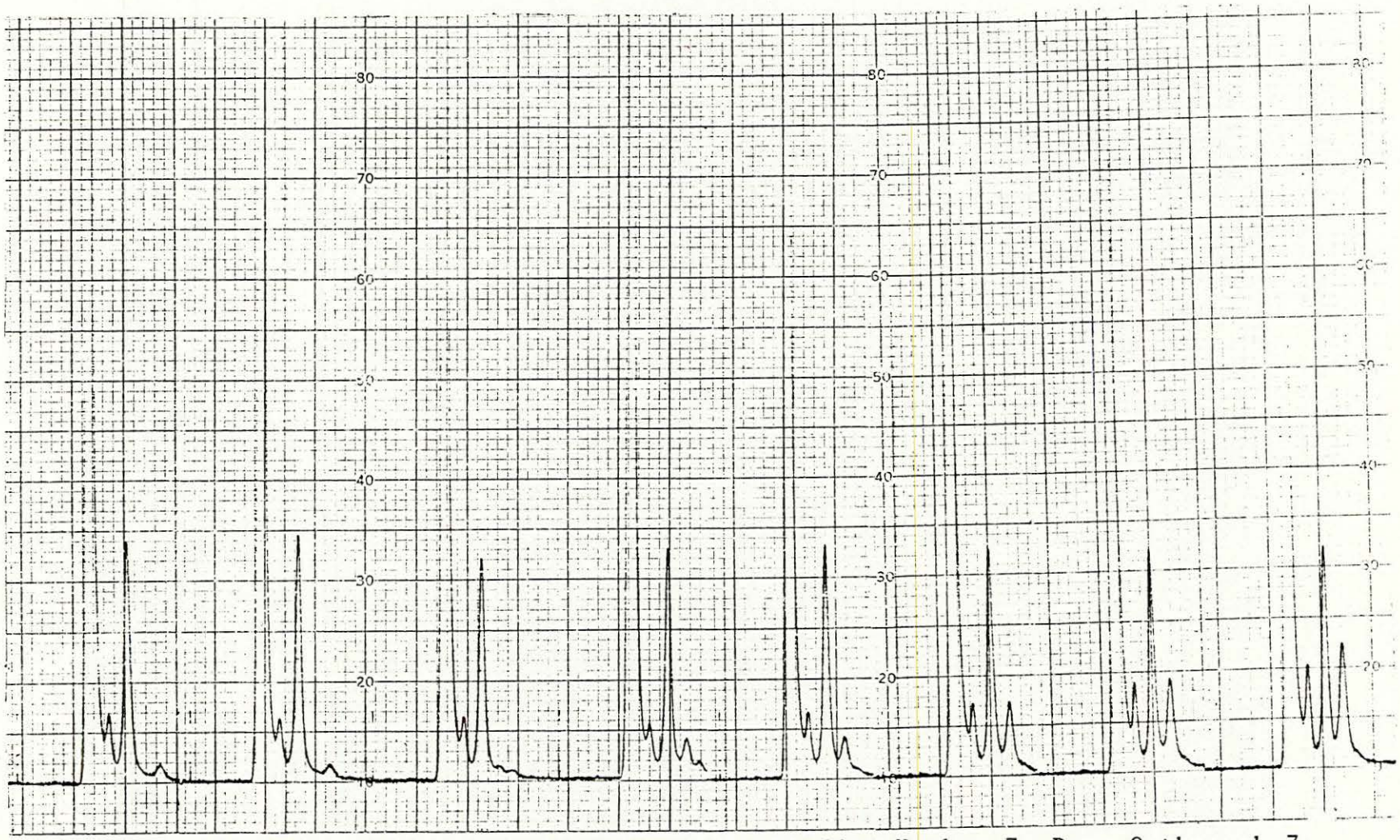

Figure 6. Cation Assays on Bile from Gall Bladder Number 7, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 1.

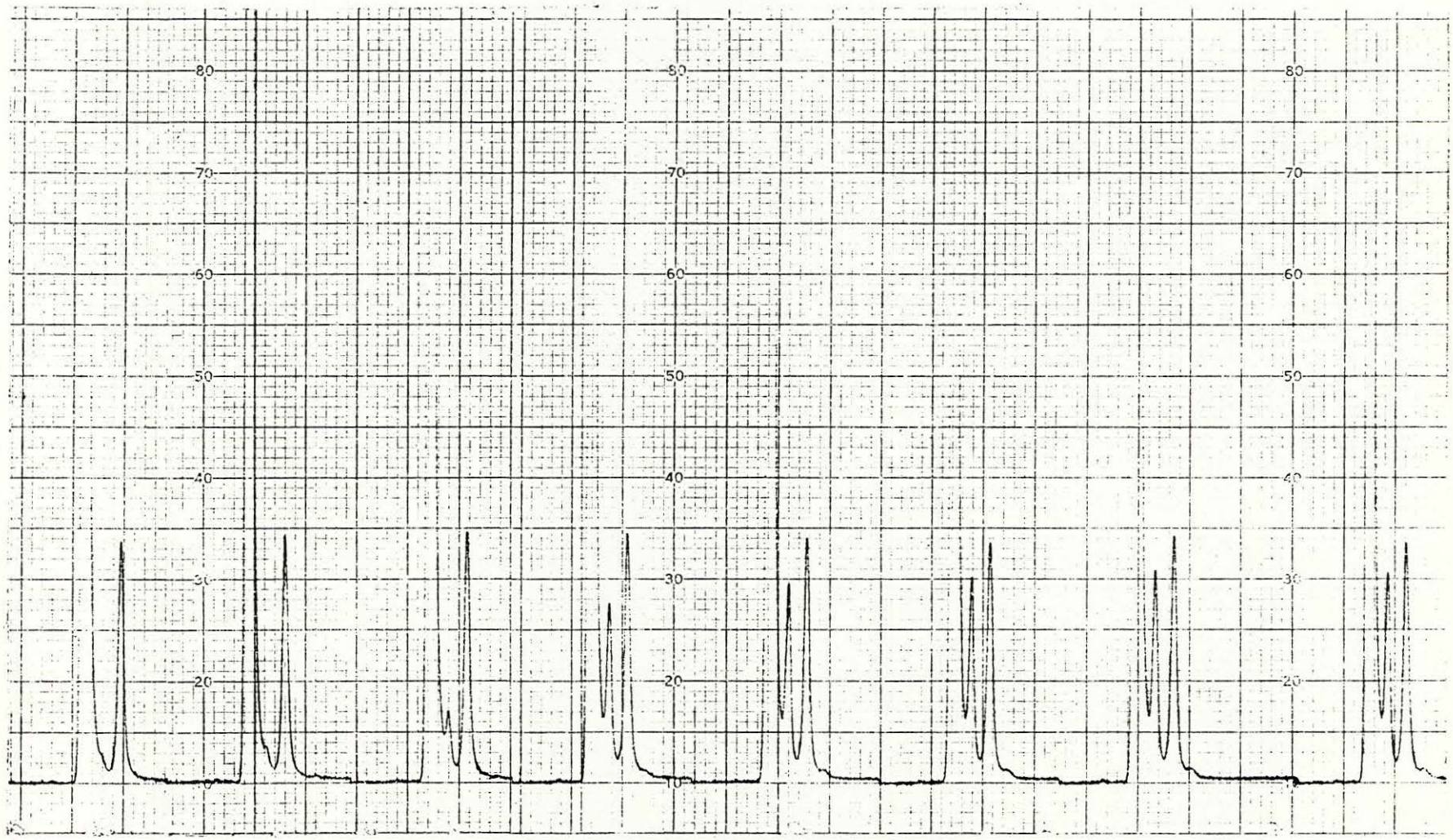

Figure 7. Cation Assays on Bile from Gall Bladder Number 8, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 1.

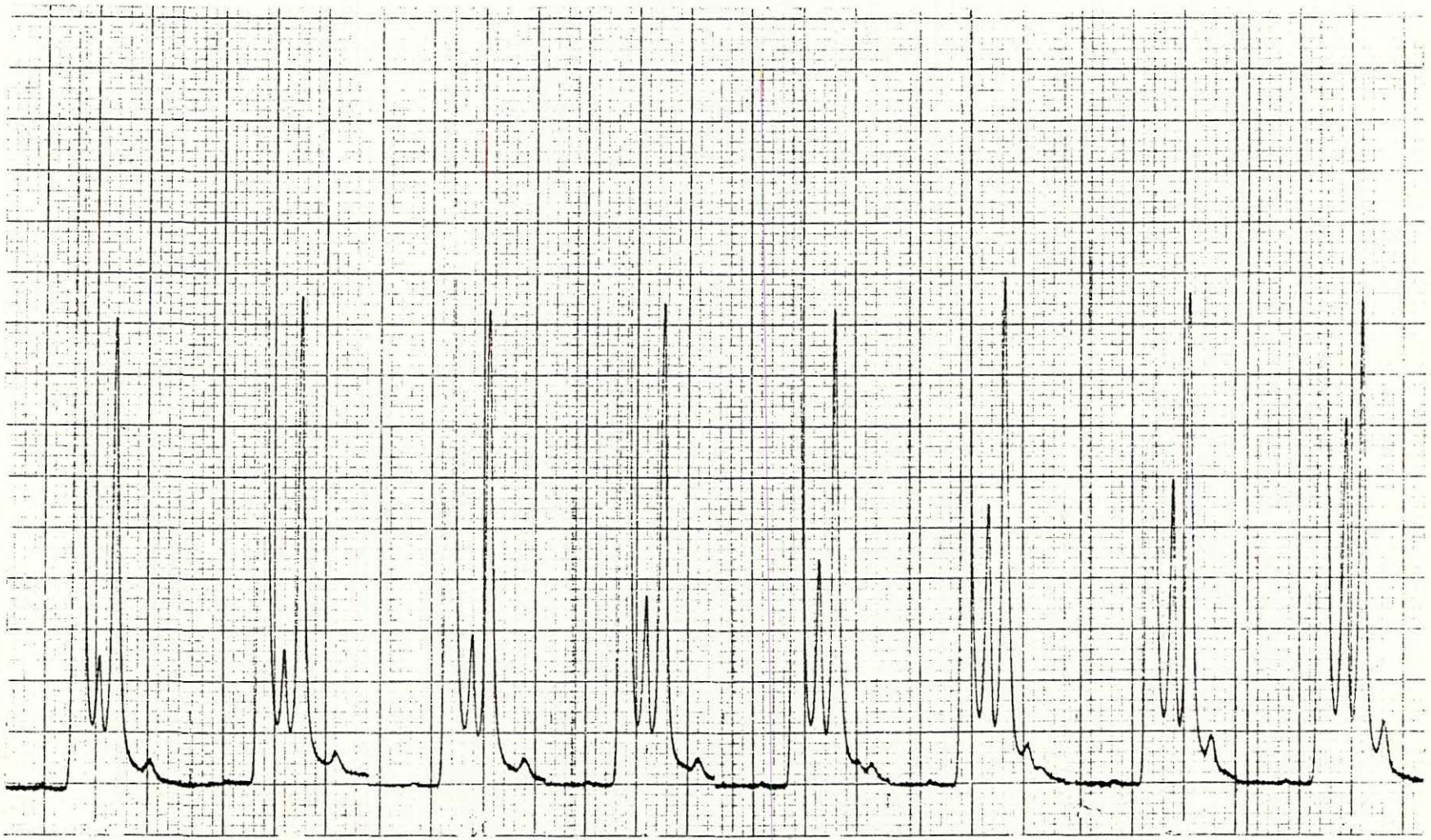

Figure 8. Cation Assays on Bile from Gall Bladder Number 9, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 1.

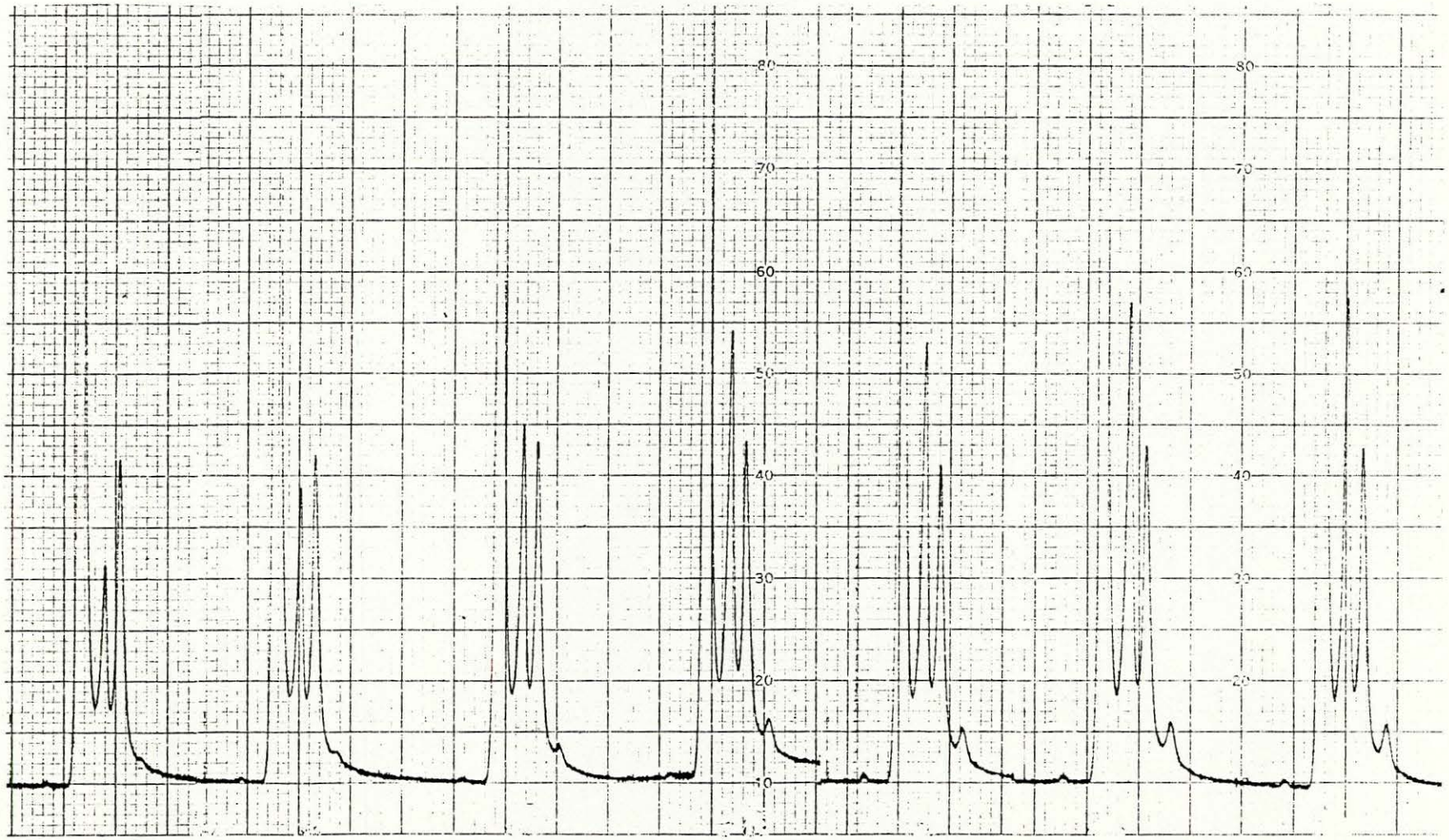

Figure 9. Cation Assays on Bile from Gall Bladder Number 10, Days 0 through 2 and 4 through 7 of Room Temperature Incubation. See caption for Figure 1.

Figure 10. Cation Assays on Bile from Gall Bladder Number 11, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 1.

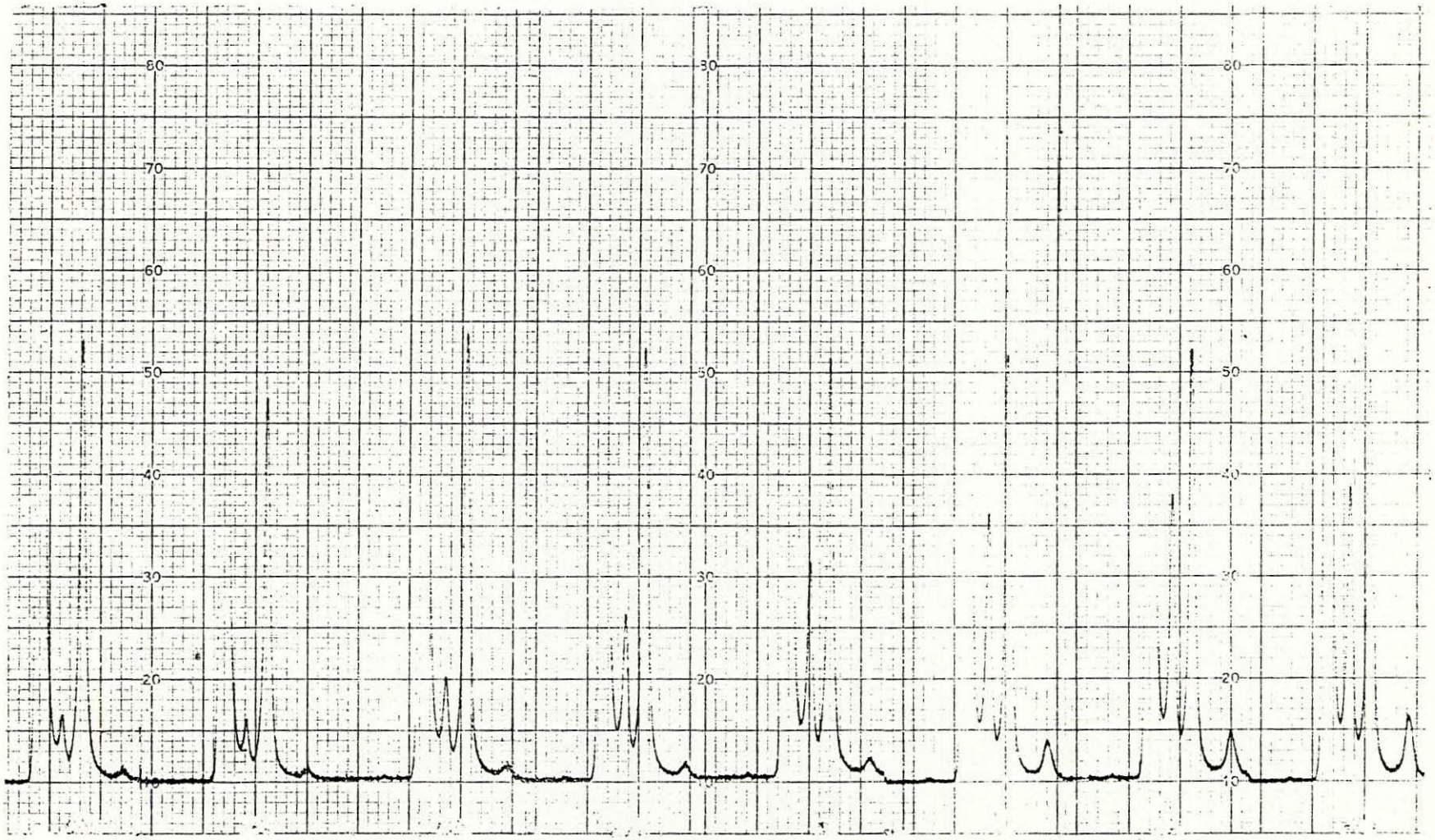

Figure 11. Cation Assays on Bile from Gall Bladder Number 12, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 1.

Figure 12. Anion Assays on Bile from Gall Bladder Number 2. Days 0 through 7 of Room Temperature Incubation. An event marker indicates sample injection (I) beginning each chromatogram. Volume front (V) may be visible. Peaks in order of appearance are A, acetate; B, unidentified (not well-separated from A); and C, formate. Small peak following formate is unidentified.

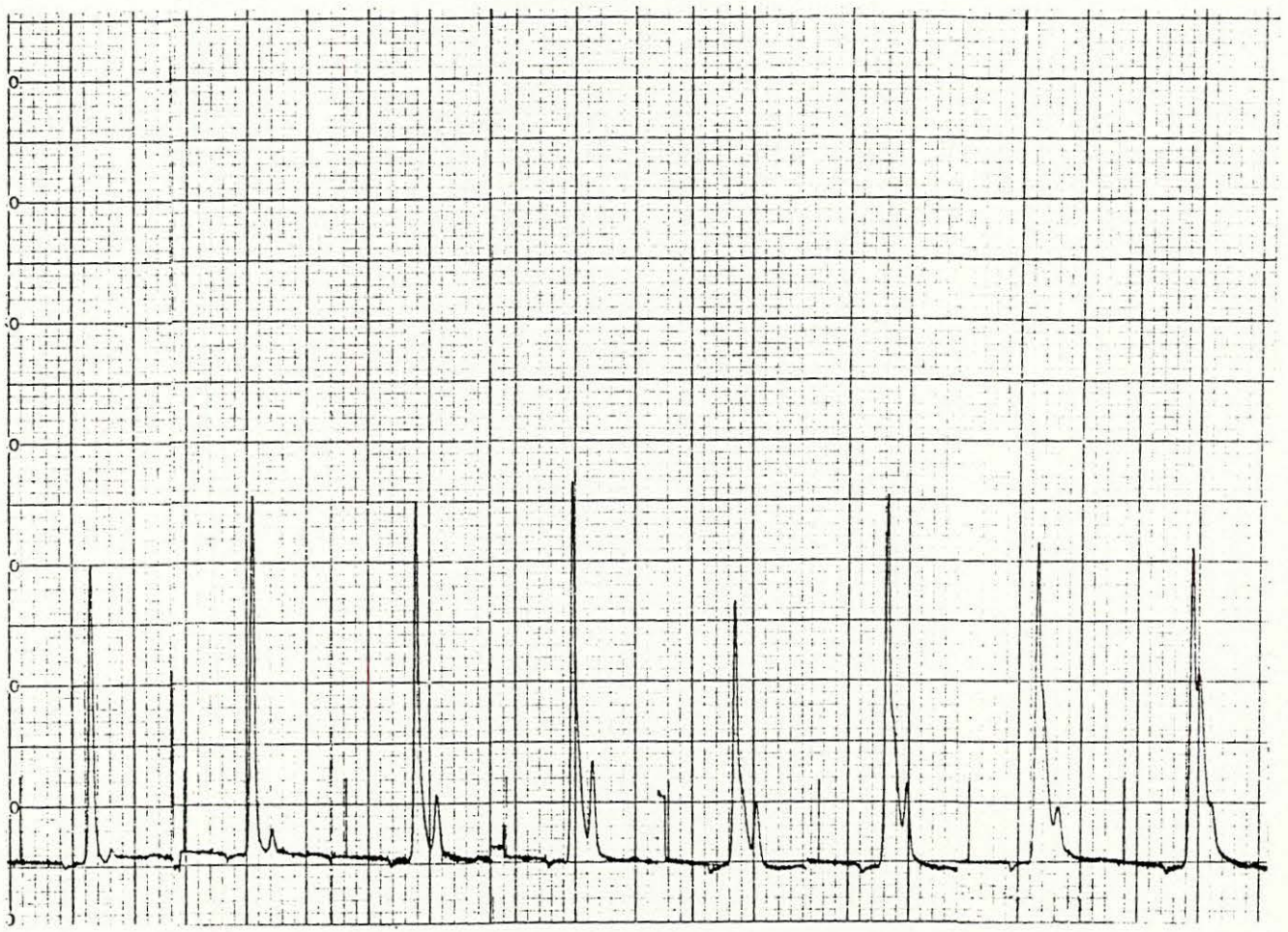

Figure 13. Anion Assay on Bile from Gall Bladder Number 3, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 12.

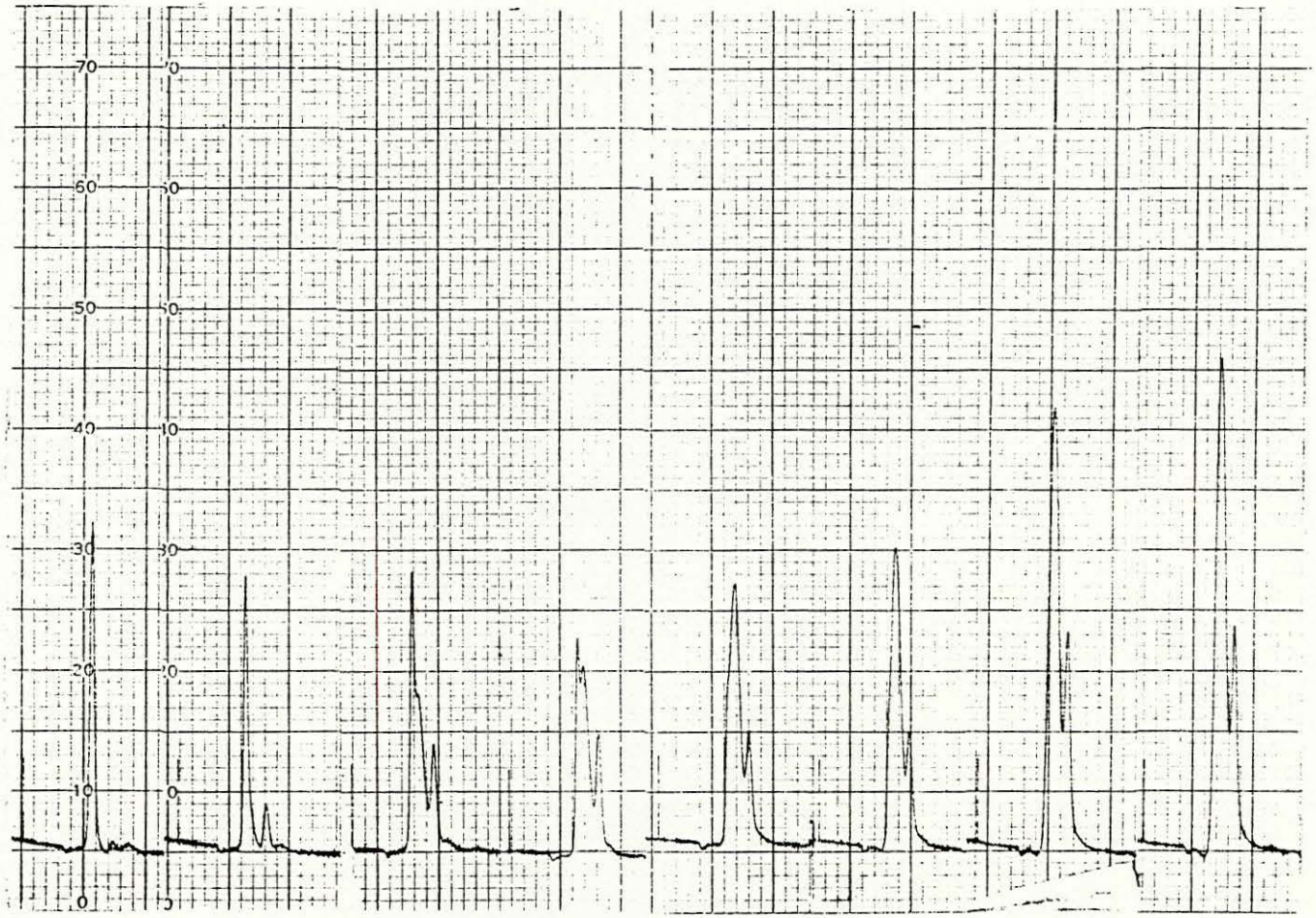

Figure 14. Anion Assays on Bile from Gall Bladder Number 4, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 12.

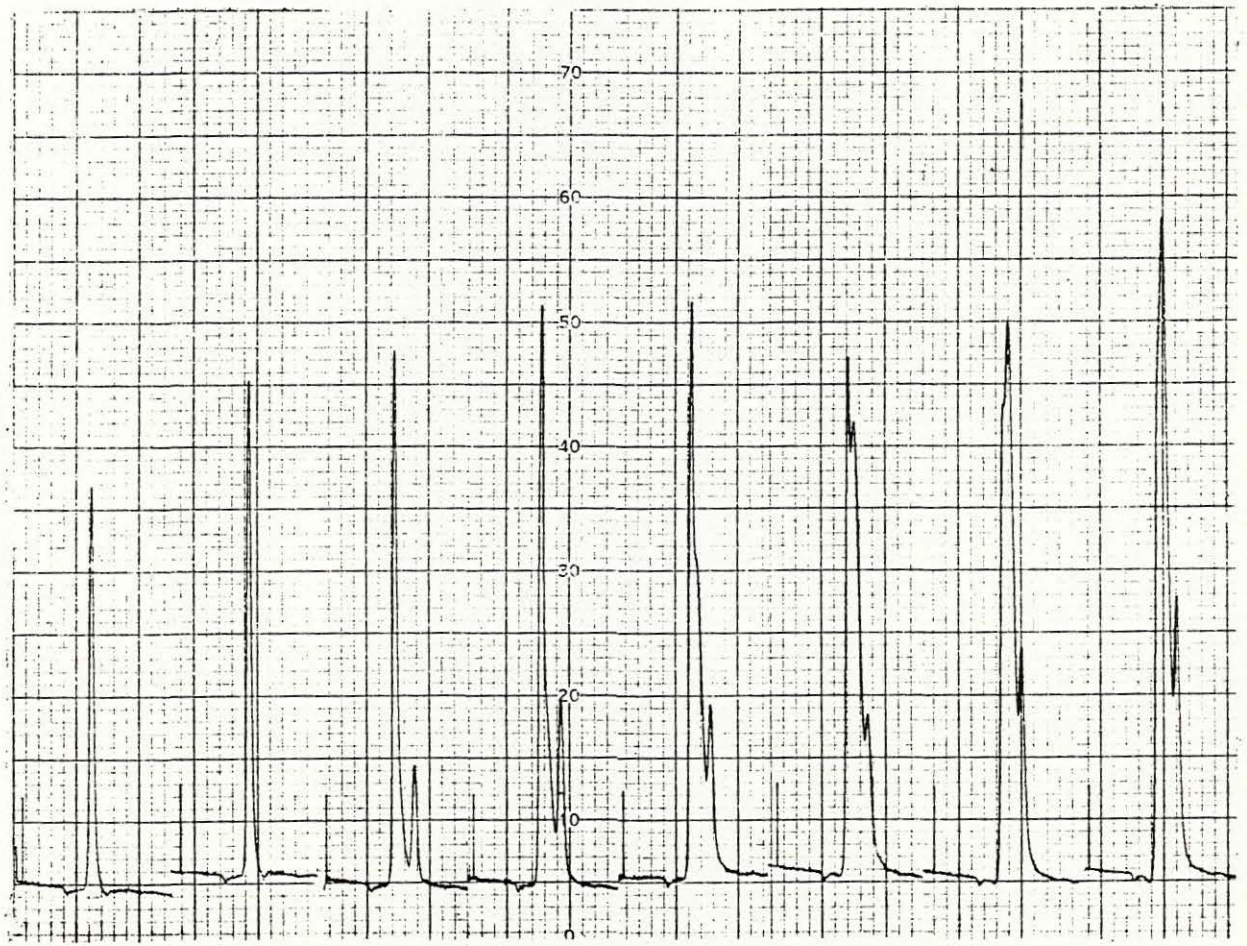

Figure 15. Anion Assays on Bile from Gall Bladder Number 5, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 12.

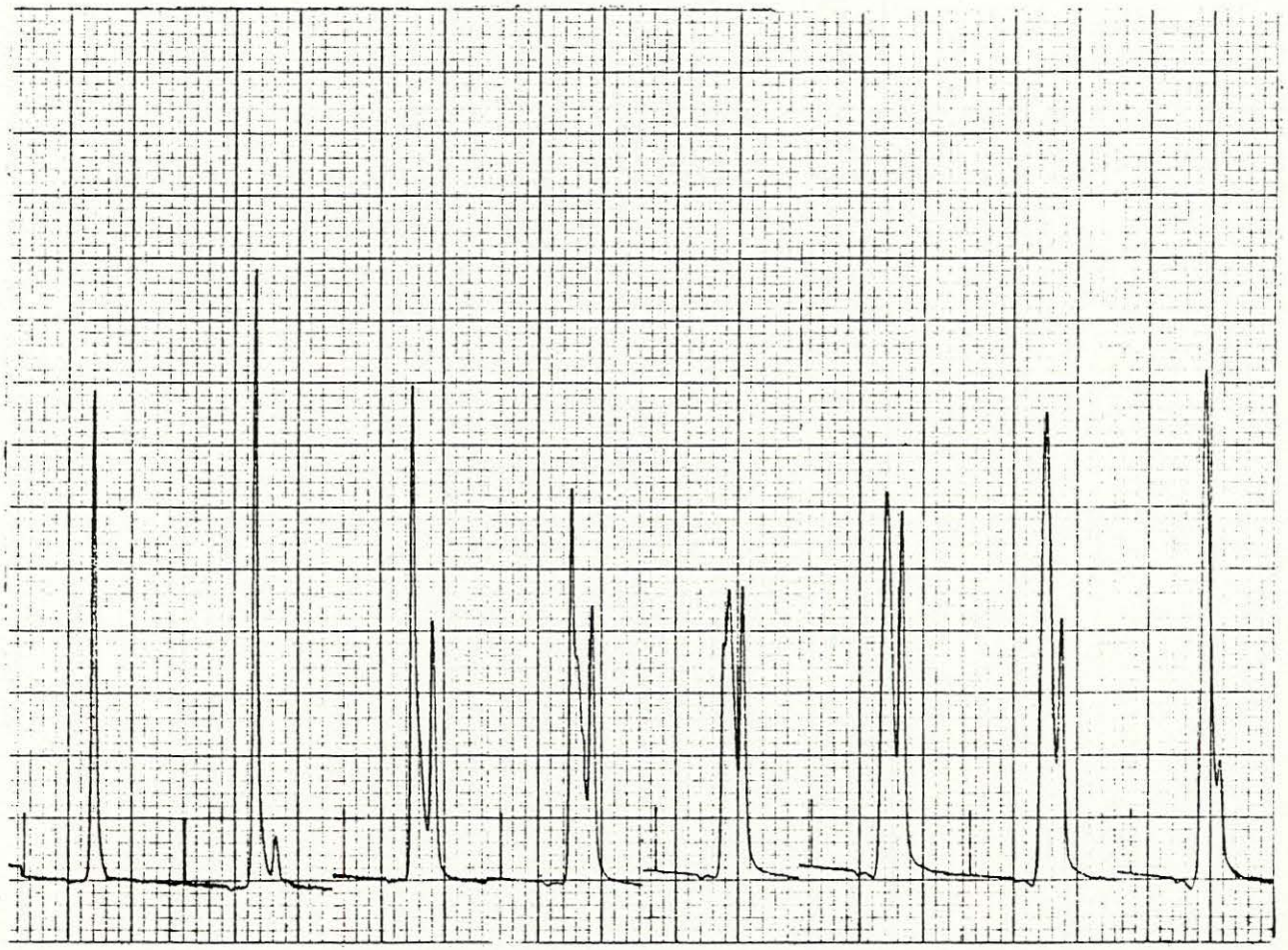

Figure 16. Anion Assays on Bile from Gall Bladder Number 6, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 12.

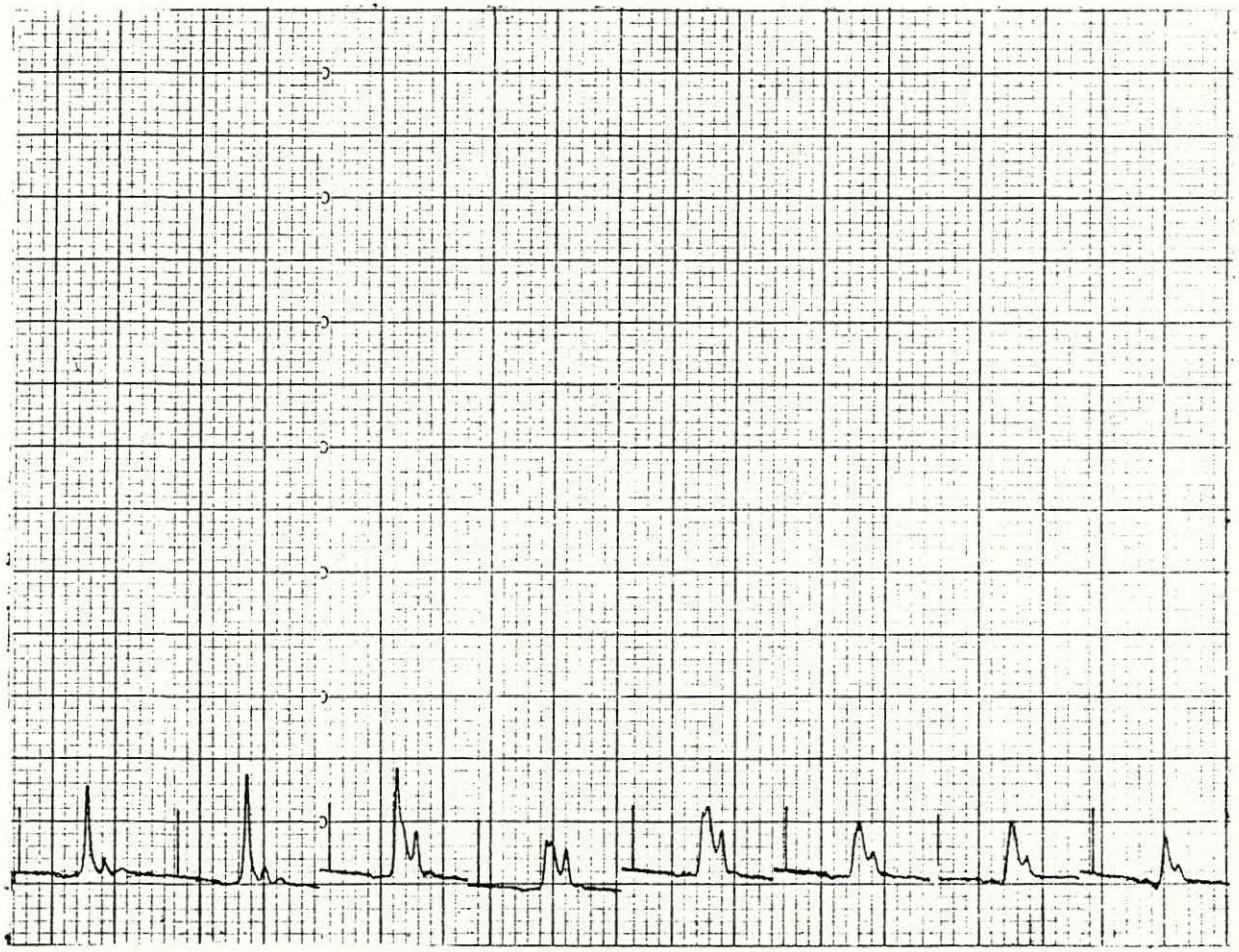

Figure 17. Anion Assays on Bile from Gall Bladder Number 7, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 12.

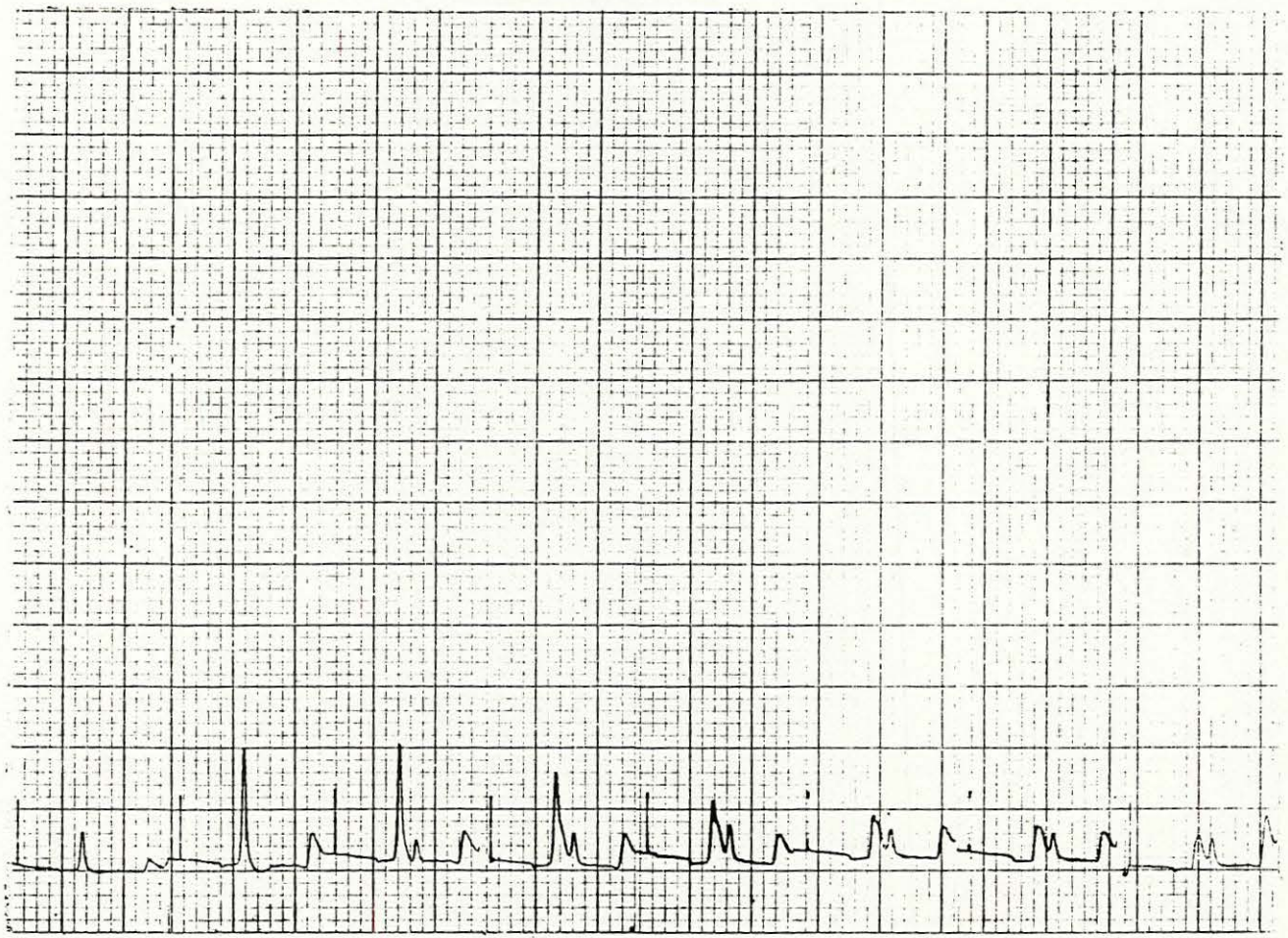

Figure 18. Anion Assays on Bile from Gall Bladder Number 8, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 12.

Figure 19. Anion Assays on Bile from Gall Bladder Number 9, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 12.

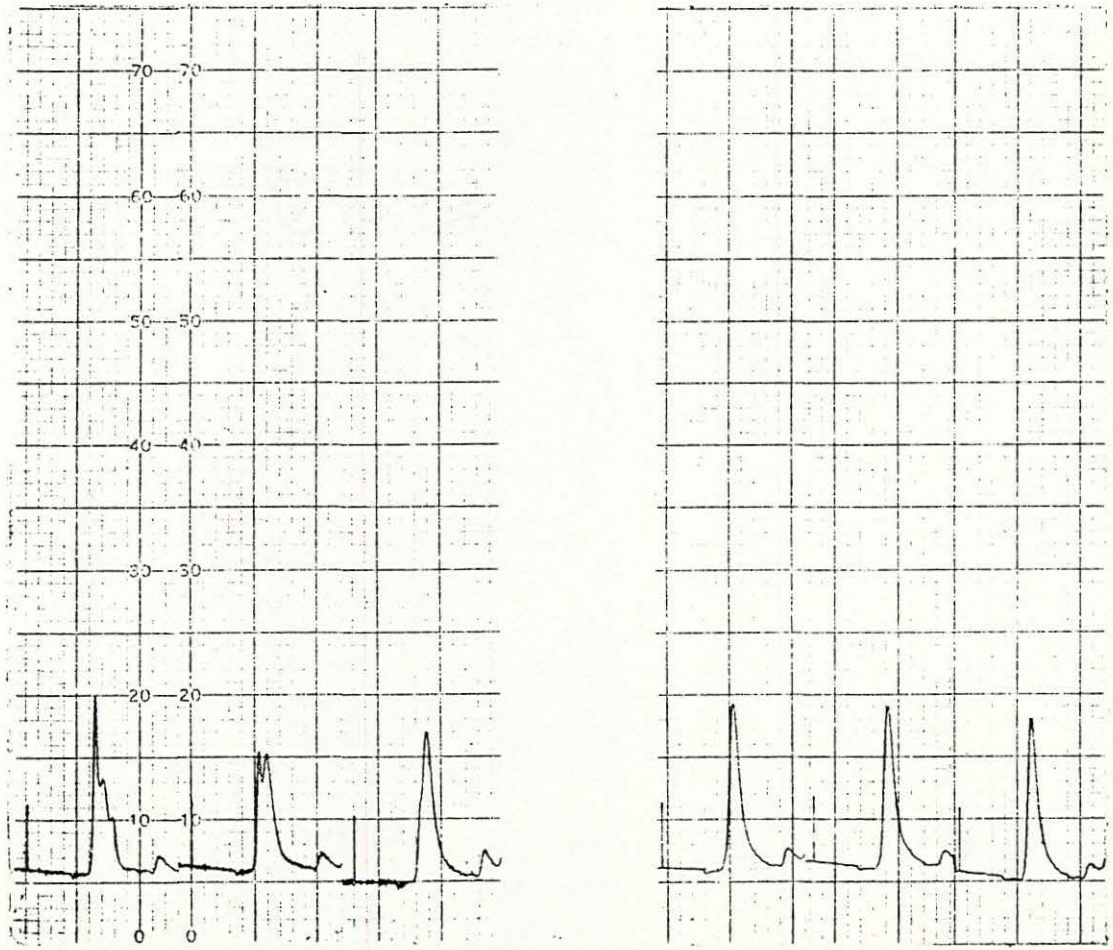

Figure 20. Anion Assays on Bile from Gall Bladder Number 10. Days 0 through 2 and 4 through 6 of Room Temperature Incubation. See caption for Figure 12.

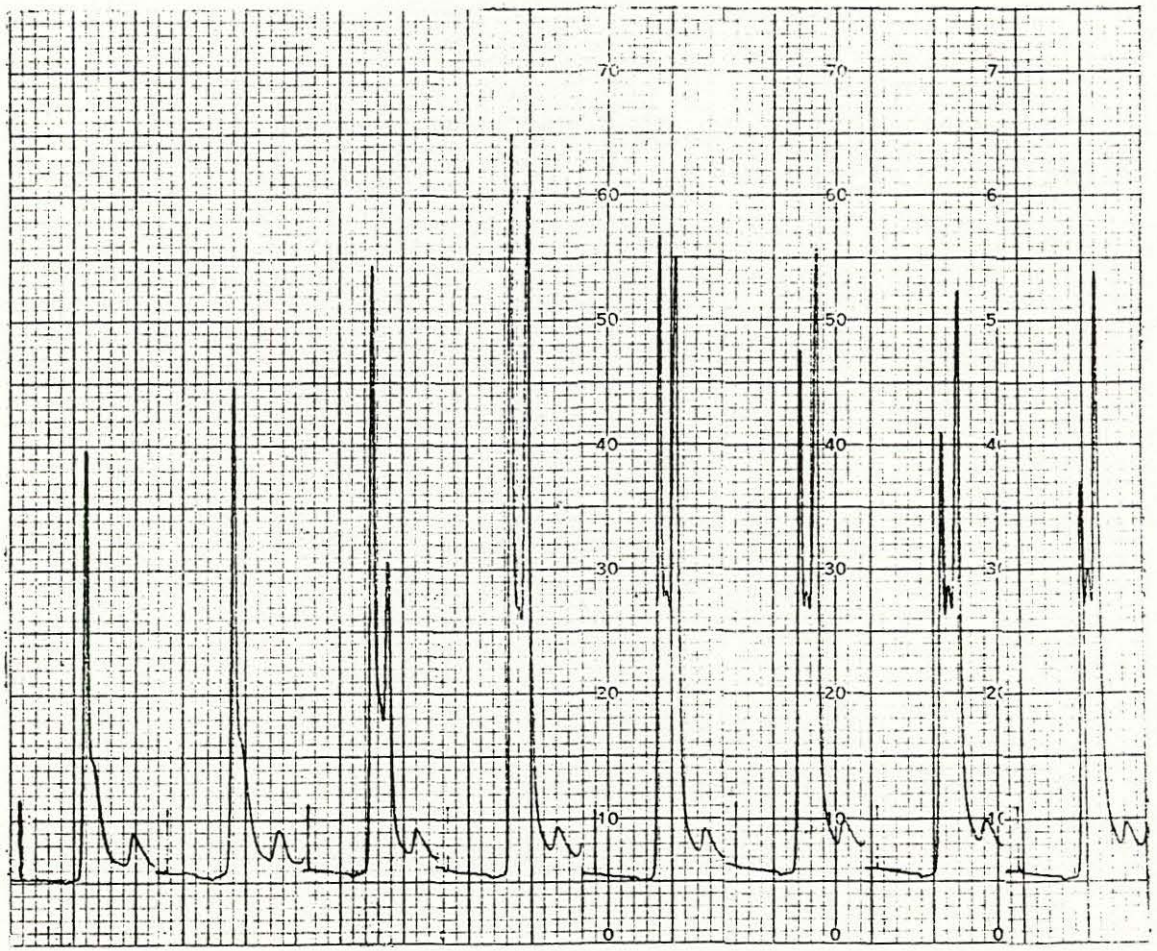

Figure 21. Anion Assays on Bile from Gall Bladder Number 11, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 12.

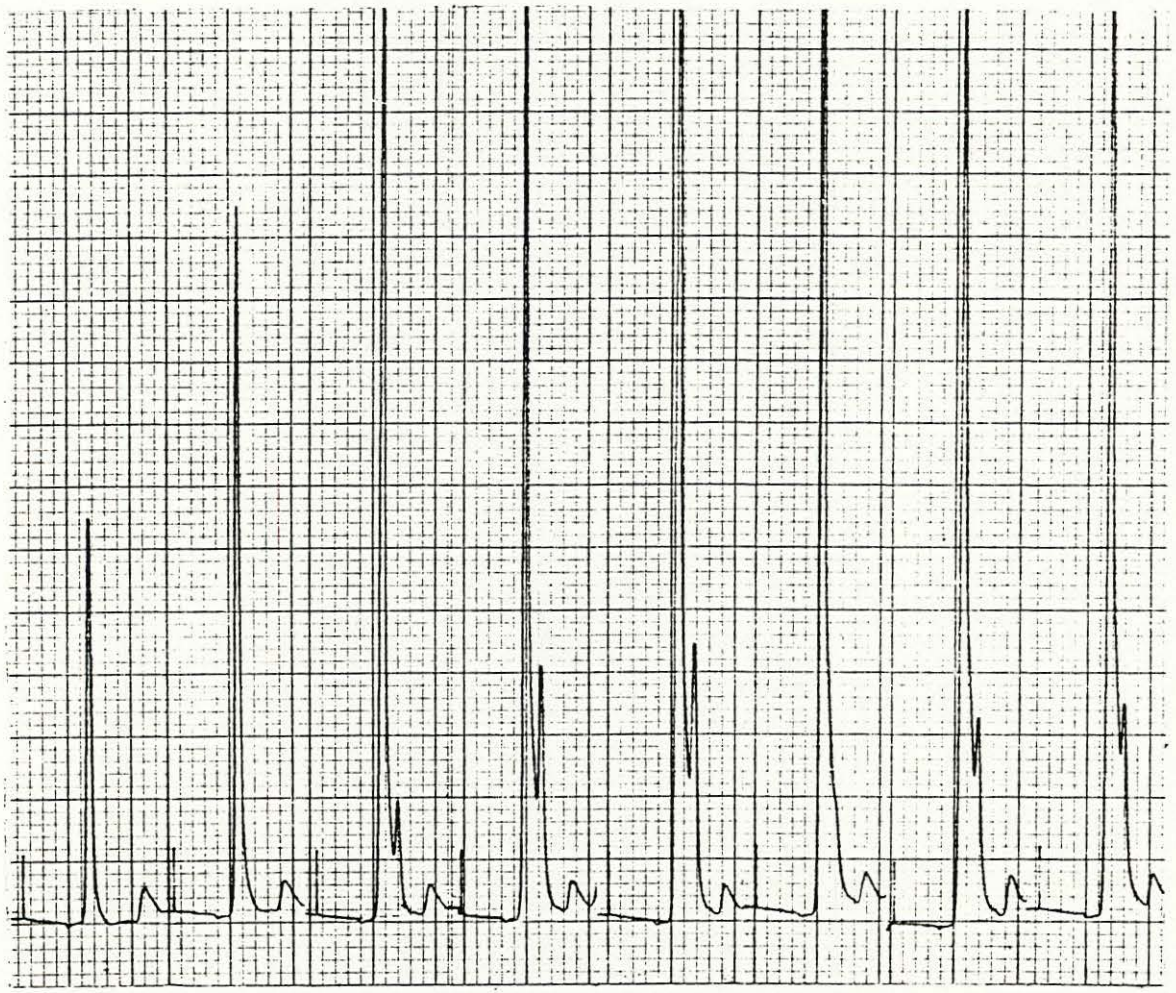

Figure 22. Anion Assays on Bile from Gall Bladder Number 12, Days 0 through 7 of Room Temperature Incubation. See caption for Figure 12.

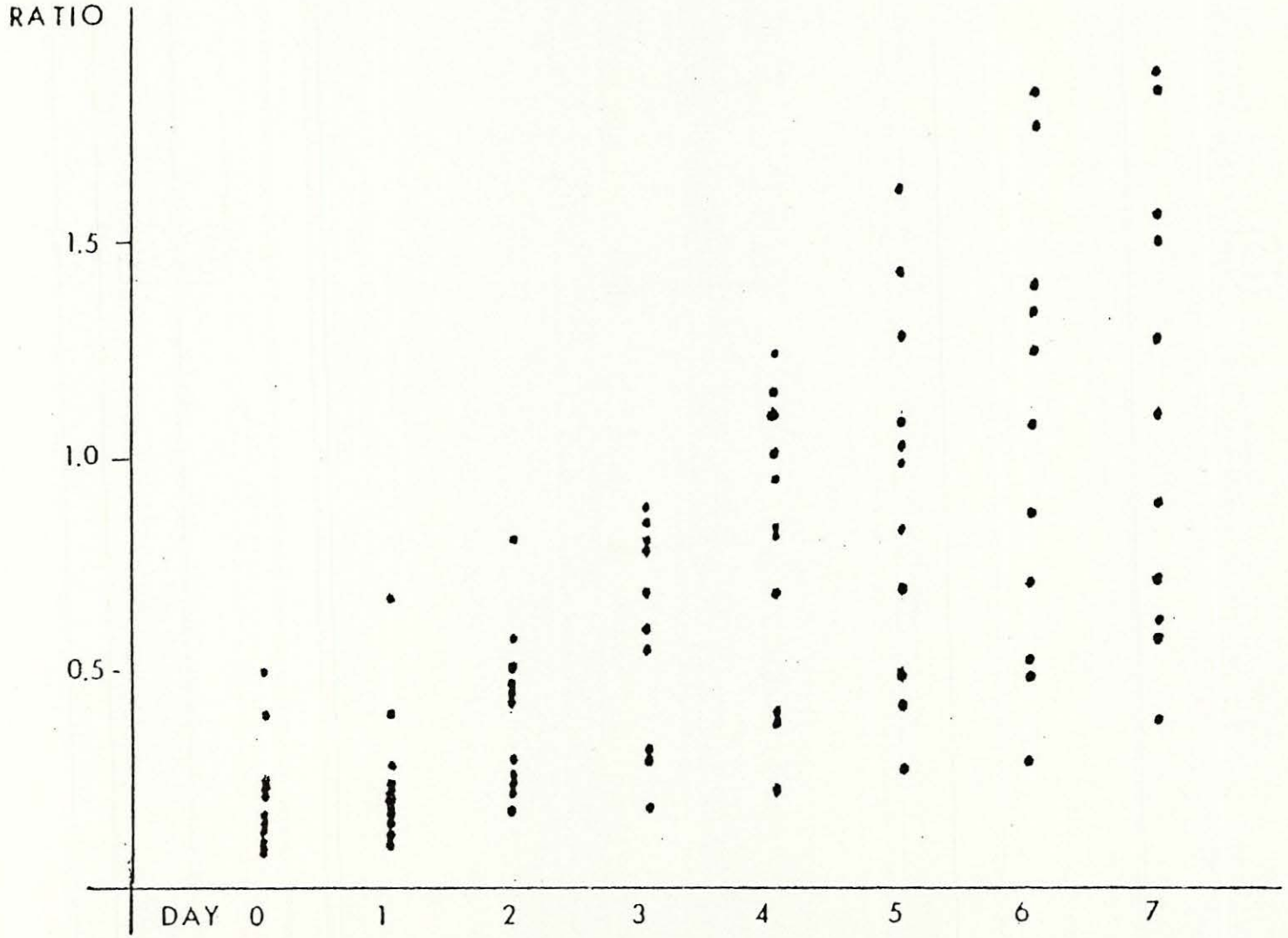

Figure 23. Ratios of Ammonium and Potassium Ion Concentrations in Incubated Gall Bladders as a Function of Time.

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